INVESTIGATION OF THE SUITABILITY OF AQUATIC INVERTEBRATES AS BIOLOGICAL INDICATORS FOR DETECTING THE PRESENCE OF *CAMPYLOBACTER SPP.* IN RECREATIONAL WATER SUPPLIES

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ATCC	American type culture collection
bp	base pairs
BHI	brain heart infusion medium
CCDA	*Campylobacter* charcoal differential (deoxycholate) agar
CFU	colony forming units
cm	centimetre(s)
dH_2O	distilled water
ddH_2O	double distilled water
DNA	deoxyribonucleic acid
dNTP	2'-deoxy nucleotide triphosphate
ESR	Institute of Environmental Science and Research Limited
EtBr	ethidium bromide
g	gram(s)
GBS	Gullain Barré syndrome
h	hour(s)
ha	hectares
Kb	kilobase pairs
Kda	kilodaltons
Km	kilometres
Kpa	kilopascals
L	litre(s)
m	metres
mm	millimetre(s)
MHB	Mueller Hinton blood medium
min	minute(s)
MPN	most probable numbers
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PVC	polyvinyl chloride
ppt	parts per thousand
PFGE	pulsed-field gel electrophoresis
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
sec	seconds
spp.	species
Tet	tetracycline
µl	microlitre(s)
µg	microgram(s)
µm	micrometre
UPMGA	unweighted pair group algorithm averages
UV	ultra-violet
V	volts
xg	times gravity

ABSTRACT

Campylobacter species, the most frequent cause of acute bacterial gastroenteritis in humans, are of worldwide significance. Drinking and recreational surface waters have been identified as a major vehicle of pathogenic *Campylobacter spp.* transmission to humans. Outbreaks of campylobacteriosis as well as sporadic cases have been reported both locally and internationally. However, in only a small proportion of cases has the responsible organism been isolated from the implicated water source. The inherent difficulties with isolation of *Campylobacter spp.* from environmental water sources make the development of more reliable, alternative isolation and detection methods a preferred option. In this study, the potential for aquatic invertebrates to act as biological indicators for detecting *Campylobacter spp.* from recreational water was investigated. The suspension and filter feeding strategies of aquatic invertebrates allowing them to concentrate *Campylobacter* cells to readily detectable levels within their tissues was explored. Sampling of water, mussels, cockles and snails from the Avon-Heathcote rivers and estuary revealed low isolation rates with no apparent correlation between *Campylobacter* presence in water and the invertebrates. Placement of freshwater mussels in the Avon and Heathcote rivers proved successful for isolation of *Campylobacter spp.*, while marine mussels, cockles and snails routinely tested negative. Hence, the freshwater mussel was chosen as a candidate bio-indicator. *In vitro* (tank) experiments showed close correlation between *Campylobacter* detection in mussels and water. However, isolation of *Campylobacter* from mussels was deemed to be no better than testing the water itself. Environmental *Campylobacter* isolates were analysed by resistotyping, *flaA* PCR-RFLP typing and PFGE to determine relationships between isolates. Although very few isolates were found to be clonal, a high level of genetic relatedness was determined between isolates from the Avon and Heathcote rivers. This indicates a common source of input between these rivers, most likely to be from birds. A validation study for the use of the *cadF* virulence gene as a marker for PCR detection of pathogenic *Campylobacter* species was under taken. Although useful for distinguishing *Campylobacter spp.* in pure culture, this system was found to show a degree of unreliability for specific detection from environmental samples.

Chapter I

Introduction

1.1 Overview of *Campylobacter* species

Since their initial isolation in 1909, members of the genus *Campylobacter*, etiological agents of acute bacterial gastroenteritis, have emerged as globally significant human pathogens (Blaser et al., 1983).

1.1.1 Taxonomy of *Campylobacter* species

Campylobacter species belong to the family *Campylobacteraceae* which is further divided into four closely related genera: *Campylobacter, Helicobacter, Arcobacter* and *Wolinella* (Corry et al., 1995; Nachamkin, 1995). Recently a closely related fifth genus '*Sutterella*' has been described (Wexler et al., 1996-cited in Engberg et al., 2000). *Campylobacter spp.* were originally designated as belonging to the genus *Vibrio* but differ significantly both biochemically and genetically from type species of this genus (Faoagali, 1984). This is particularly evident in terms of DNA base composition and preference for reduced oxygen growth conditions (Corry et al., 1995). The name *Campylobacter* is derived from the Greek words *Campylo* meaning 'curved' and *bacter* meaning 'rod' (Faoagali, 1984). The genus *Campylobacter* comprises 18 species and sub-species, which possess heterogenous properties (Nachamkin, 1995). Of this group of *Campylobacter* species, four 'thermophilic' species, *C. jejuni, C. coli, C. lari* and *C. upsaliensis,* are most often associated with enteric disease (Ketley, 1995).

1.1.2 *Campylobacter* morphology

Campylobacter spp. are usually curved, spiral or S-shaped rods (Allos and Blaser, 1995). Organisms are actively motile by means of a single, polar, unsheathed flagellum at one or both ends facilitating a 'corkscrew-like' motion (Nachamkin, 1995). An exception to this

basic cell morphology is the recently described species '*C. showae*', which appears as straight rods with multiple flagella (Nachamkin, 1995). *Campylobacter* species are small, Gram-negative and non-spore forming with a size range of 0.2-0.9μm in width and 0.5-5μm in length (Nachamkin, 1995). Most species are microaerophilic, requiring an atmosphere containing 3-15% oxygen and 3-10% carbon dioxide, along with temperatures of 37-43°C for optimum growth (Ketley, 1995; Corry et al., 1995). Under specific environmental conditions such as exposure to atmospheric oxygen or prolonged stationary phase growth in dry, relatively warm conditions, the typical helical cell morphology undergoes metamorphosis to a spherical or coccoid form (Allos and Blaser, 1995; Ketley, 1995; Corry et al., 1995). This morphological transition is associated with the onset of a change in culturability from viable, culturable to a viable but non-culturable (VNC) state (Rollins and Colwell, 1986).

1.1.3 Genetics of *Campylobacter spp.*

The chromosome of *Campylobacter spp.* is relatively small, ranging from about 1.6-1.8Mb for *C. jejuni* and *C. coli,* with *C. upsaliensis* having the largest chromosome at approximately 2.0Mb (Taylor, 1992a; Ketley, 1995). The chromosome is circular and highly A-T rich with a G-C content of 32-35% (Nuijten et al., 1990; Ketley, 1995). In comparison, *Escherichia coli* has a chromosome of 4.6Mb with a 50% G-C content (Ketley, 1995). The small chromosome size of *Campylobacter spp.* is consistent with requirements for specialised growth media, inability to ferment carbohydrates or degrade complex substances and biochemical inertness (Taylor, 1992a). Extra-chromosomal elements such as conjugative plasmids and bacteriophages have been reported in *Campylobacter spp.* and could play an important role in diversity of metabolism (Ketley, 1995, Jankovic, 1999).

1.1.4 Emergence as a pathogen

Campylobacter spp. were initially isolated and described in the early 1900s, with *Vibrio fetus*, now *Campylobacter fetus*, the first member associated with infertility, foetal infection and abortion in cattle and sheep (The National Advisory Committee on Microbiological Criteria for Foods, 1995). *Campylobacter* species were considered to be

almost exclusive veterinary pathogens until the 1970s (Ketley, 1995). However, isolations from humans were reported as early as 1947 when *Campylobacter fetus* was isolated from a pregnant woman who had a septic abortion (Allos and Blaser, 1995). Subsequently, *Campylobacter spp.* were occasionally isolated from blood, cerebrospinal fluid (CSF) and other body fluids of severely immunocompromised patients (Allos and Blaser, 1995; Ketley, 1995). In 1957, these organisms were first postulated to be a common cause of human diarrhoea and were successfully isolated from human faeces in the late 1960s (Blaser et al, 1983). It was not until the early 1970s that selective stool culture techniques were developed that allowed the recognition of *Campylobacter* species as a common cause of acute enterocolitis in humans (Allos and Blaser, 1995, Ketley, 1995; The National Advisory Committee on Microbiological Criteria for Foods, 1995). *C. jejuni* and *C. coli* have subsequently been recognised as the most important human pathogens within *Campylobacter* with *C. jejuni* responsible for up to 90% of diarrhoeal disease (campylobacteriosis) caused by *Campylobacter* species (Blaser et al., 1983; Ketley, 1995). In New Zealand, campylobacteriosis was made a notifiable disease in 1980 and a steady rise in notifications has occurred since, with dramatic peaks in 1993 (McNicholas et al., 1995; Eberhart-Phillips, 1995) and 1998 (Figure 1.1). Campylobacteriosis is now the most frequently notified communicable disease in New Zealand (Brieseman, 1990; Eberhart-Phillips et al., 1997; Withington and Chambers, 1997).

1.1.5 Incidence of campylobacteriosis

The incidence of campylobacteriosis in New Zealand is one of the highest reported for the developed world. In 1981, a national average of 14 notified cases per 100 000 population was reported (Brieseman, 1990). By 1998 this incidence rate had increased by more than 20 times to 317 per 100 000 (The New Zealand Public Health Report, 1999-cited in Mitchell, 1999). Since 1993, campylobacteriosis notifications have consistently exceeded 200 cases per 100 000 population per annum (Withington and Chambers, 1997). The increase in reported campylobacteriosis cases between 1980 and 1998 is shown in Figure 1.1.

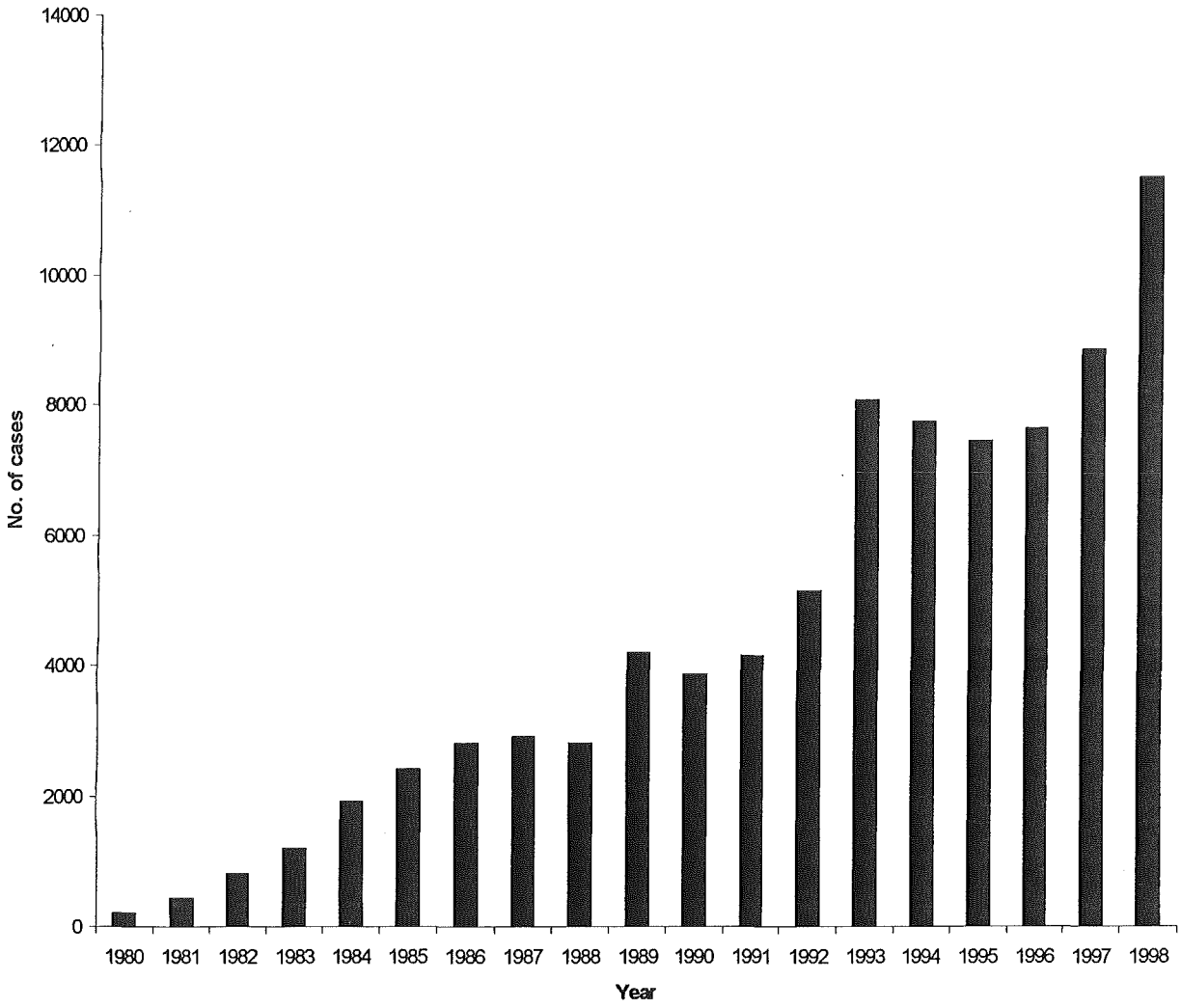

Figure 1.1: Campylobacteriosis notifications in New Zealand from 1980 to 1998 (Adapted from New Zealand Public Health Reports)

In recent years, the Canterbury provincial area has routinely reported infection rates significantly higher than the national average (Brieseman, 1994). In 1992, a rate of 297 cases per 100 000 population was recorded for Christchurch compared to the New Zealand national average of 152 per 100 000 (Ikram et al., 1994, Lane and Baker, 1993). Similarly, in 1993 when a nationwide increase in campylobacteriosis of 56% was reported at 238 per 100 000, cases within the Canterbury area health board sharply increased to 445 cases per 100 000 (McNicholas et al., 1995). The epidemiology of campylobacteriosis in Canterbury was found to be similar to other areas in New Zealand

and thus the reasons for the consistently higher rates remains as yet unclear (Brieseman, 1994). A survey addressing the possibility of changes in laboratory diagnostic procedures as being responsible for the nationwide increase in campylobacteriosis found that laboratory culture methodologies had not changed and hence could not account for the higher annual infection rates (McNicholas et al., 1995). However, a greater number of patients presenting with illness at medical centres coupled with more specific testing for *Campylobacter* species at medical laboratories could account for part of the increase in incidence (Mitchell, 1999).

Campylobacteriosis incidence in New Zealand is consistently higher than that of other industrialized countries. The 1992 rate of 152 notifications per 100 000 was twice that of England and three times the reported cases from Australia or Canada (Eberhart-Phillips, 1995). The reported incidence of campylobacteriosis for England and Wales between 1989 and 1997 has ranged from 80-97 cases reported per 100 000 clearly indicating a less rapid increase in incidence when compared to New Zealand (Skirrow, 1990; Adak et al, 1995; Mitchell, 1999). In Norway, incidences of 1.8-13.1 per 100 000 were reported between 1979 and 1988 while 14-86 per 100 000 were reported in New Zealand over the same period (Brieseman, 1990; Kapperud and Aasen, 1992). In the United States, total food poisoning cases have been estimated at between 24 and 81 million cases with up to 10 000 deaths each year (Maurice, 1994). With respect to campylobacteriosis, an annual reported incidence of 5-6 per 100 000 persons has been the steady rate of isolation since 1982 when the Center for Disease Control and Prevention (CDC) began surveillance (Allos and Blaser, 1995; The National Advisory Committee on Microbiological Criteria for Foods, 1995). Campylobacteriosis is not a notifiable disease in the United States however, and more intensive reporting from population-based studies have estimated the annual incidence to be as high as 1000 cases per 100 000 persons (Blaser, 1997).

The true incidence of infection in most cases is grossly underestimated by laboratory isolations (Allos and Blaser, 1995). This is due to a number of factors, such as the infected person not seeking medical care, physicians not all requesting stool cultures or microbiology laboratories not specifically testing for *Campylobacter* species (Allos and

Blaser, 1995). In addition, limitations of direct culture methodologies may result in *Campylobacter* species being present but not cultured or the isolation of *Campylobacter* may not be reported to the health department.

1.1.6 Epidemiology of *Campylobacter* infections

The reported cases of campylobacteriosis in New Zealand show a distinct seasonal trend. Notifications increase over the spring and summer months peaking usually in December. Significantly lower reporting occurs during the winter months (see Figure 1.2) (Brieseman, 1990; Lane and Baker, 1993).

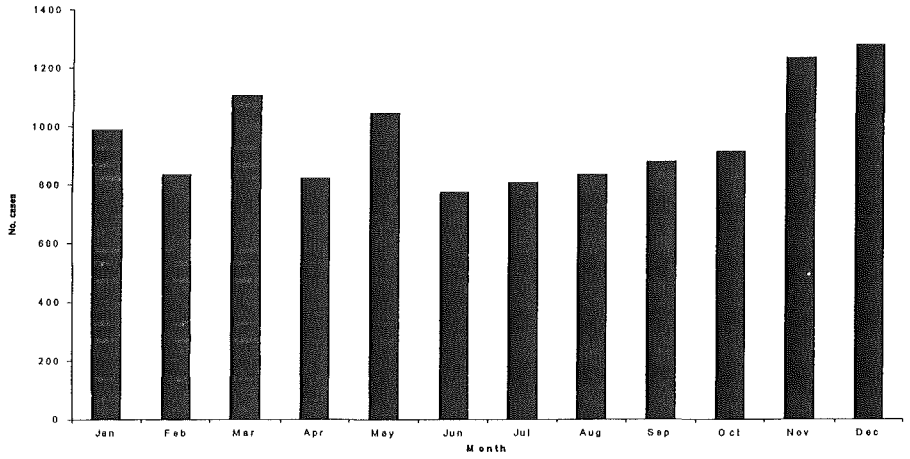

Figure 1.2: Seasonal fluctuations in campylobacteriosis notifications for Canterbury during 1998

This phenomenon has also been observed overseas. In England and Wales, a strikingly consistent rise that begins in May, reaches a peak in July and falls steadily to base levels by December is observed (Skirrow, 1990). Similar patterns of seasonal fluctuation have also been observed in Norway and the United States (Blaser, 1997; Kapperud and Aasen, 1992). In direct contrast to campylobacteriosis cases, the environmental isolation rates of *Campylobacter* are higher in the cooler months of winter and autumn (Brennhovd et al., 1992; Koenraad et al., 1994). The environmental isolation and survival of *Campylobacter* species are addressed in chapters II and III of this thesis.

The incidence of campylobacteriosis also shows a bimodal age distribution. Isolation rates are highest in infants 1-4 years of age with a second peak in young adults between the ages of 15-30 years (Skirrow, 1990; Eberhart-Phillips et al., 1995; Blaser, 1997). The reason for the second peak in adults is unclear (Lane and Baker, 1993) but it has been suggested that the popularity of 'fast' takeaway food among young people is a contributing factor (Skirrow, 1990). In all age categories, males are disproportionately represented with an annual notification rate significantly higher than that of females (Lane and Baker, 1993). Notification rates of 323 per 100 000 and 274 per 100 000 were reported in 1993 for males and females in New Zealand respectively (1.17:1) (Lane and Baker, 1993). Recently, the New Zealand ratio has increased slightly to approximately 1.2:1 (J. Klena, personal communication). In overseas studies, male to female ratios of 1.7:1 have been reported for campylobacteriosis incidence (Skirrow, 1990).

In New Zealand, reported rates of campylobacteriosis are five times higher among persons of European origin (320 per 100 000) than among Maori (64 per 100 000) or Pacific Islanders (59 per 100 000) (Lane and Baker, 1993; Eberhart-Phillips et al., 1995). The reason for these differing rates is also unclear. Generally, rural areas show higher rates of infection than urban areas and outbreaks have often been implicated with contamination of drinking water supplies (Brieseman, 1987; Brieseman, 1994). Among occupations that pose a health risk of campylobacteriosis are meat workers and meat handlers (Brieseman, 1994).

The high incidence of campylobacteriosis in New Zealand is reported to have considerable financial impact on the country. In 1995, Withington and Chambers (1997) estimated the financial cost of campylobacteriosis to be $4.48 million when the costs of hospital admissions, visits to general practitioners and loss of work were combined. In Britain, a cost of £273 per patient was estimated, which totalled over £9 million per year (Skirrow, 1990). The true costs of campylobacteriosis have been estimated to be up to ten times higher than the above values if non-reported cases are also taken into consideration (Skirrow, 1990; Withington and Chambers, 1997). A conservative estimate

of 20 000 working days lost per year due to *Campylobacter* infections has been suggested (Faoagali, 1984).

The possible sources of sporadic *Campylobacter* infections as well as outbreaks have been extensively studied. In 1995, the multi-centre analysis of gastroenteritis induced by *Campylobacter* (MAGIC) study representing the first large scale epidemiological national survey of New Zealand *Campylobacter* infections, found the consumption of raw or undercooked foods (notably poultry and unpasteurised dairy products) to be associated with disease. Recent overseas travel, contact with animals and the consumption of untreated water were also important risk factors for *Campylobacter* infection (Eberhart-Philips, 1995). Currently, the consumption of contaminated chicken is considered the single most important risk factor for contracting a *Campylobacter* infection, with many sporadic cases linked to a recent chicken meal (Brieseman, 1990; Skirrow, 1990; Lane and Baker, 1993; Ikram et al., 1994; Allos and Blaser, 1995; Eberhart-Phillips et al., 1997).

The next largest transmission source implicated for *Campylobacter* transmission is the ingestion of untreated drinking and surface waters (Brieseman, 1994; Adak et al., 1995). A number of major gastroenteritis outbreaks from water due to *C. jejuni* have been reported (Kramer et al., 1996). Many have been attributed to contamination of local water supplies such as those present at campgrounds and in small communities (Sacks et al., 1986; Stehr-Green, 1991; Bohmer, 1997) while outbreaks from swimming in recreational waters have also been reported (Cabelli et al., 1982; Corbett et al., 1993; Drenchen and Bert, 1994). The isolation of *Campylobacter* species from environmental waters has resulted in considerable interest in recreational water supplies as transmission vehicles of *C. jejuni* (Carter et al., 1987; Pearson et al., 1993; Koenraad et al., 1997). A major complicating factor with water-borne transmission of *Campylobacter* infections is the inconsistent isolation of the source of illness from implicated water supplies.

1.1.7 Clinical symptoms of campylobacteriosis

Upon ingestion of *C. jejuni* or *C. coli*, a spectrum of illness can be observed ranging from asymptomatic to fulminant sepsis and death (Nachamkin 1995; Allos and Blaser, 1995). Illness often begins with one to two days of premonitory symptoms such as headache, myalgias, arthralgias and backache (Blaser et al., 1983). The most common clinical manifestation of a *Campylobacter* infection is acute abdominal pain with fever and malaise, which progresses to profuse diarrhoea and vomiting (Nachamkin, 1995; Ketley, 1995; The National Advisory Committee on Microbiological Criteria for Foods, 1995). The diarrhoeic stools of infected patients often contain fresh blood (in 25% of patients), muco-pus and an inflammatory exudate with leucocytes (Nachamkin, 1995; Ketley, 1995). At this stage of infection, rapidly motile *Campylobacter* cells may be observed by microscopy of fresh faeces (Ketley, 1995). The typical gastrointestinal symptoms produced by *Campylobacter* species are clinically indistinguishable from those caused by some viruses or by other enteric bacterial pathogens such as *Salmonella* and *Shigella* species (Blaser et al., 1983; Allos and Blaser, 1995).

Occasionally, the symptoms of campylobacteriosis are confused with other medical conditions such as inflammatory bowel disease or acute appendicitis with unnecessary appendectomies sometimes being performed prior to diagnosis of *Campylobacter* infection (Blaser et al., 1983; Allos and Blaser, 1995). The onset of symptoms can occur within one to seven days after ingestion of *Campylobacter* cells with illness persisting for up to three weeks. Usually, acute diarrhoea lasts for two to three days and is accompanied by exhaustion and dehydration (Ketley, 1995). Abdominal pain and discomfort can continue after the cessation of diarrhoea and patients can remain culture positive for *Campylobacter* from faeces for several weeks after symptoms have resolved (Ketley, 1995). *Campylobacter* infections are usually self-limiting and a complete recovery is apparent in less than seven days without specific antimicrobial therapy (Allos and Blaser, 1995). However, relapses or prolonged severe illness can occur in up to 20% of all cases, although relapses are often less severe than the initial attack (Blaser et al., 1983; Ketley, 1995).

The bacterial dose required for campylobacteriosis onset is relatively low when compared to other enteric bacteria. Human volunteer studies have shown that diarrhoeal illness can be induced by ingestion of as low as 800 cells of *C. jejuni* while higher doses increased the rate of infection (Black et al., 1992). In a buffered solution such as milk, only 500 organisms have been reported to cause diarrhoeal illness in a human volunteer (Robinson, 1981-cited in Faoagali, 1984). The specific strain of *Campylobacter* ingested also appears to have a bearing on the severity of illness as well as the individuals susceptibility to the organism (Black et al., 1992; Wallis, 1994). It would appear that virulence is also, to a certain extent, species-specific, with *C. jejuni* represented in the majority of cases. However, *C. upsaliensis* has also been isolated from stool specimens of campylobacteriosis patients and has been implicated as an unrecognised cause of human diarrhoea (Goosens et al., 1990).

The self-limiting nature of *Campylobacter* infections negates the need for widespread use of antibiotic therapy. In most cases, oral rehydration and correction of electrolyte imbalances is all that is required (Allos and Blaser, 1995). However, antibiotic treatment has been shown to reduce the duration of *Campylobacter* excretion in stools with patients exhibiting prolonged or worsening symptoms benefiting the most from such treatment (Allos and Blaser, 1995). *C. jejuni* and *C. coli* are susceptible to a variety of antimicrobial agents including macrolides, fluoroquinalones and aminoglycosides (Nachamkin, 1995). Erythromycin has been the drug of choice for treatment of most *C. jejuni* gastrointestinal infections (Nachamkin, 1995). This agent is characterised by low toxicity, a narrow spectrum of activity and low cost (Allos and Blaser, 1995). The resistance of *C. jejuni* to erythromycin is less than 5% with little change in this resistance rate over the last 15 years (Nachamkin, 1995, Allos and Blaser, 1995). Erythromycin resistance rates of *C. coli* have been shown to vary considerably with up to 80% resistance reported in some studies (Nachamkin, 1995). However, erythromycin stearate is incompletely absorbed and has a local effect throughout the bowel in addition to its systemic effects (Allos and Blaser, 1995). Hence, ciprofloxacin has been used as an alternative drug treatment. The effectiveness of ciprofloxacin however has been

somewhat decreased since the emergence of ciprofloxacin-resistant *Campylobacter* strains (Nachamkin, 1995).

1.1.8 Extraintestinal *Campylobacter* diseases

The importance of *Campylobacter* as a human pathogen goes beyond the magnitude and costs of enteric disease, but also the potentially fatal extraintestinal diseases it is frequently associated with. The penetration of the intestinal cell wall and subsequent invasion of the blood stream is speculated to occur in 0.15% of all intestinal *Campylobacter* infections (Nachamkin, 1995). The resulting diseases include cholecystitis, pancreatis, cystitis, endocarditis, peritonitis, erythema nodosum, abortion, neo-natal sepsis and bacteraemia (Blaser et al., 1983; Allos and Blaser, 1995, Nachamkin, 1995). Approximately 0.4% of *C. jejuni* isolates reported to the CDC in the United States are obtained from blood (Allos and Blaser, 1995). In addition to *C. jejuni* and *C. coli*, bacteraemia as a result of *C. lari* infections have also been reported (de Guevara et al., 1994; Reed et al., 1998).

Post-infection complications from *Campylobacter* infections have been known to occur, often with severe consequences. Haemolytic anaemia, encephalopathy, stroke, acute motor axonal neuropathy (AMAN) and Guillain-Barré syndrome (GBS) are all post infection complications that have been associated with *Campylobacter* species (Allos and Blaser, 1995, Rees et al., 1993).

In addition to campylobacteriosis, GBS, an acute demyelinating disease of the peripheral nervous system, is the most commonly associated disease with *Campylobacter* infections. AMAN and GBS are clinically indistinguishable and *C. jejuni* has been recovered from 20-40% of patients approximately one to three weeks before the onset of any neurological symptoms (Allos and Blaser, 1995; Buzby et al., 1997). Since the decline of polio, GBS has become the most common cause of acute neuromuscular paralysis with an annual incidence of 1-2 cases per 100 000 in many parts of the world (Blaser et al., 1997; Buzby et al., 1997; Nachamkin, 1997, Wassenaar et al, 2000). GBS is especially prevalent in northern China, Mexico, Spain and Korea (Blaser et al., 1997). Typical

symptoms of disease progression include weakness followed by dysesthesias in the legs which subsequently spreads to the arms and upper body (Blaser et al., 1997). Approximately 70% of patients make a complete recovery, although severe irreversible neurological damage, the need for ventilatory assistance and death can result (Blaser et al., 1997).

Similar to campylobacteriosis cases, GBS follows a number of comparable trends. It is most prevalent in the summertime, it affects people of any age and is more common among males (Blaser et al., 1997). These correlations between campylobacteriosis and GBS give further strength to the association of *C. jejuni* with this neurological disorder. Studies addressing the possible connections between *C. jejuni* serotypes and GBS have found that certain *C. jejuni* serotypes pose an increased risk of GBS (Wassenaar et al., 2000). *C. jejuni* strains of the Penner-HS:19 serotype appear to be the most commonly isolated strain from GBS patients although other strains such as *C. jejuni* HS:41 have also been implicated (Nachamkin, 1995; Wassenaar et al., 2000).

1.1.9 Pathogenesis of *Campylobacter* species

The specific mechanisms of *Campylobacter* pathogenesis are not well understood. However, three main mechanisms of pathogenesis have been proposed:

The first mechanism of pathogenesis has been described as the penetration and proliferation of *Campylobacter spp.* within the intestinal epithelium resulting in bloody diarrhoea (The National Advisory Committee on Microbiological Criteria for Foods, 1995). Upon entering the intestine via the stomach, *C. jejuni* initiates infection by adhering to the mucosal surfaces of the gastrointestinal tract (Wallis, 1994). *C. jejuni* is thought to use its motility and spiral shape for projection through mucosal surfaces, coupled with the production of adhesins for colonisation of the intestine (Wallis, 1994; Ketley et al., 1996). The flagella may play an integral part in the process of mucosal colonisation (Ketley et al., 1996). Proliferation of *C. jejuni* within the intestinal epithelial cells results in cellular death, which has the clinical manifestation of bloody diarrhoea.

The second mechanism of pathogenesis is the penetration of the intestinal mucosa with cell proliferation in the lamina propria and mesenteric lymph nodes (The National Advisory Committee on Microbiological Criteria for Foods, 1995). It has been suggested that invasiveness in *C. jejuni* can be induced by the presence of other entero-invasive bacteria or viruses (Wallis, 1994). This allows translocation of *C. jejuni* to the mesenteric lymph nodes which may produce an enteric fever without frank dysentery or instigate bacteraemia (Cotran et al., 1994-cited in Mitchell, 1999; Wallis, 1994).

The third and most controversial pathogenic mechanism is the induction of disease by production of a toxin resulting in watery diarrhoea. Isolates of *C. jejuni* are known to produce at least two exotoxins, these are the heat-labile cytotoxin and the cytolethal distending toxin (Pickett and Whitehouse, 1999; Fragoso et al., 1996; Musmanno et al., 1998). The *C. jejuni* enterotoxin is thought to attach to ganglioside receptors on the plasma membrane of enterocytes of the intestinal mucosa. From there it enters cells and interacts with intracellular targets such as adenylate cyclase resulting in raised cyclic AMP levels. This causes disruption to normal ion transport mechanisms resulting in fluid being drawn from cells to cause watery diarrhoea (Wallis, 1994). The presence of the cytolethal distending toxin in *C. jejuni* is thought to invoke the arrest of intestinal cells in the G2 phase of the cell cycle, causing cells to increase to five times their normal size and disintegrate (Pickett and Whitehouse, 1999). The induction of enterotoxins for the pathogenesis of *Campylobacter* species is controversial however, with the expression levels of toxins considered to be too low to be of clinical relevance and antibodies against toxins not usually detected in patient's sera suggesting toxins may not be principally involved (Wasssenaar, 1996).

1.1.10 Vaccines against *Campylobacter* species

Evidence from epidemiologic and volunteer studies have shown that development of a vaccine to prevent gastrointestinal disease and limit colonisation is possible (Scott, 1997). Two major target populations for vaccines have been reported. These are humans and chickens (Kaijser and Meinersmann, 1996). It is believed that for a vaccine to be effective against an enteric agent, it must be able to stimulate intestinal immunity (Scott,

1997). It is for this reason that an oral route of immunisation has been identified as the preferred approach (Scott, 1997). Two major types of vaccines for *Campylobacter* have been considered. Firstly, the live attenuated vaccines which have been trialed against two bacterial pathogens (*Salmonella typhi* and *V. cholerae*) and shown to effectively stimulate mucosal immunity and provide excellent results in field or volunteer challenge studies (Kaijser and Meinersmann, 1996; Scott, 1997). However, the limited knowledge of *Campylobacter* pathogenesis has prevented such a vaccine from being made available (Scott, 1997).

Secondly, the oral sub-unit vaccines, which require administration with an adjuvant in order to generate appropriate mucosal immune responses, have been considered (Kaijser and Meinersmann, 1996). The lack of a conserved protective antigen has limited the development of this type of vaccine (Scott, 1997). Current progress with respect to the development of a vaccine is promising with some optimism that vaccines are likely to be effective against intestinal *Campylobacter* infections (Kaijser and Meinersmann, 1996).

1.2 Research Model

Contaminated water has been shown to be an important vehicle for the transmission of *Campylobacter spp.* to humans resulting in both large outbreaks and sporadic cases. However, in only a small proportion of cases have the causative *Campylobacter* organism been isolated from the implicated water source (Blaser and Cody, 1986).

1.2.1 Central research question

Given the difficulties experienced in establishing the presence of *Campylobacter spp.* in water supplies, the use of accurate indicator organisms or an alternative method of detection is preferable. Therefore the principal question addressed in this study is:

'Are aquatic invertebrates useful biological indicators for detecting the presence of human pathogenic Campylobacter species in recreational waters?'

1.2.2 Research hypothesis

There are a number of possible and probable sources of *Campylobacter* contamination of aquatic environments. Figure 1.3 represents a simplistic model of the major input sources of *Campylobacter spp.* into recreational waters and the proposed accumulation by aquatic invertebrates. The four major *Campylobacter* input sources are represented by birds, humans, animals and soil. Faecal inputs to water from birds and animals such as dogs, cattle and sheep, run-off and leaching of *Campylobacter* from soils and contamination by infected individuals engaging in recreational activities all result in the bacterial loading of the water. Although the *Campylobacter* inputs to water can be significant, only low numbers of organisms may be detectable. This is partly complicated by the tendency for cells to enter a viable but non-culturable state. Therefore, it is proposed that aquatic invertebrates that are present in the water may be capable of accumulating *Campylobacter* cells present in the water. Consequently, the research hypothesis for this study is:

'The suspension and filter feeding strategies of aquatic invertebrates will enable them to concentrate cells within their digestive tissues at a level that is more readily detectable by current culture methods than the surrounding water itself'

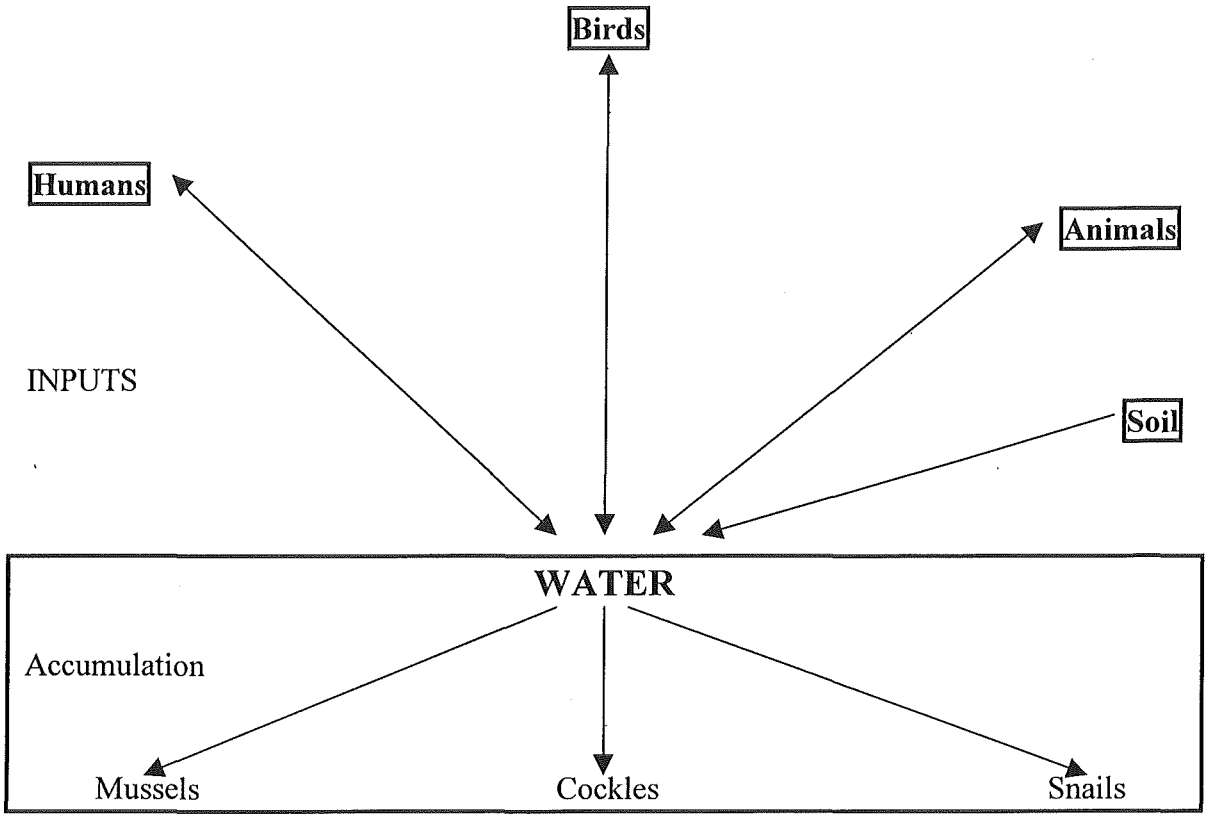

Figure 1.3: Simplistic diagram showing the major input sources of *Campylobacter* species to recreational water supplies and the proposed accumulation by aquatic invertebrates

1.2.3 Research predictions

The above hypothesis allows for the formulation of specific testable predictions:

1. For a given recreational water site to pose a public health threat, it must first harbour *Campylobacter* organisms. Therefore, the first prediction is that 'water is a reservoir (ie., a constant but not necessarily continual source of contamination) for *Campylobacter*

species'. This can be tested by sampling from chosen recreational waters and isolating potentially pathogenic *Campylobacter* species.

2. For a candidate invertebrate bio-indicator to be of epidemiological use, it must have the capabilities to obtain *Campylobacter* cells from the water and retain them for some period of time within their tissues. Hence the second prediction is that 'aquatic invertebrates are capable of accumulating *Campylobacter* cells from the water'. This prediction can be tested in two major ways. Firstly, by the successful isolation of *Campylobacter* from environmental invertebrate samples and secondly by performing *in vitro* experiments to assess whether a potential bio-indicator is capable of *Campylobacter* uptake from seeded water.

3. The suitability of a potential biological indicator is dependant on how closely the presence of *Campylobacter* relates between it and the water. The third prediction is that 'the presence of *Campylobacter* in the invertebrate closely mimics the presence of *Campylobacter* in the surrounding water'. This prediction can be tested by comparing the detection of *Campylobacter* in the candidate invertebrate bio-indicator with the detection of *Campylobacter* in the water.

4. To pose a public health threat, *Campylobacter spp.* isolated from environmental water and invertebrate samples must be able to cause disease in humans. As a result, the fourth prediction made is that '*Campylobacter* species present in water are potentially pathogenic'. Three major avenues for testing this prediction are available. Firstly, speciation of environmental isolates as those most frequently associated with disease (namely, *C. jejuni* and *C. coli*) would indicate a potential for pathogenicity. Similarly, the detection of specific virulence genes such as the *cadF* adhesin gene would further strengthen the case for pathogenic potential of isolates. Finally, the use of tissue culture experiments where epithelial cell lines are used for infection by environmental *Campylobacter* isolates, would confirm the potential pathogenicity of isolates.

5. To give epidemiological insights into the source and prevalence of environmental *Campylobacter* isolates, the final prediction that 'environmental isolates will have some degree of relatedness to each other and will have links to input sources and potentially, isolates from cases of reported campylobacteriosis' is made. A range of phenotypic and molecular typing methods can be employed for comparing isolates to test this prediction.

1.2.4 Specific Research objectives

To assess the suitability of aquatic invertebrates as biological indicators for detecting *Campylobacter* from recreational water, the following specific objectives will be addressed:

1. To sample from a range of recreational water and invertebrate types in order to test for the presence of *Campylobacter* species using direct culture methods.

2. Assess the suitability of invertebrates that consistently harbour *Campylobacter* as bio-indicators by performing *C. jejuni* uptake experiments and comparing the detection of *C. jejuni* in water with accumulation by the potential bio-indicator.

3. Validate the use of the *cadF*-PCR speciation system developed by Konkel et al. (1999a) for distinguishing *C. jejuni* and *C. coli* isolates, and use as a marker for determining virulence potential in *Campylobacter* isolates.

4. Establish the epidemiological relationships between environmental isolates by using phenotypic and molecular characterisation techniques.

1.3 Thesis Organization and Structure

This thesis has been written in chapter format. Chapter I is a general introduction to *Campylobacter* bacteriology and introduces many of the concepts dealt with in further detail in subsequent chapters. In addition, chapter I contains the research model for this study along with the specific objectives for assessing the suitability of invertebrate bio-indicators for detecting *Campylobacter*. Chapters II-V follow a general format with each having its own introduction, materials and methods, results and discussion sections. Chapter II contains the results of environmental isolation of *Campylobacter* species from water and invertebrates. Chapter III addresses the suitability of a chosen invertebrate as a biological indicator and assesses the survival of *C. jejuni* in water. Validation of the *cadF* gene as a *C. jejuni* and *C. coli*-specific marker is performed in chapter IV. Results of phenotypic and molecular typing using resistotyping, *flaA*-RFLP typing and pulsed field gel electrophoresis as well as speciation of environmental isolates are outlined in chapter V. Chapter VI is a general summary of all results obtained in this thesis and includes possibilities for future research.

Chapter II

Environmental *Campylobacter spp.* Isolation

2.1 Introduction

In order to select an appropriate candidate organism(s) for use as a biological indicator, it is important to assess the prevalence of *Campylobacter* species in aquatic environments and their correlation with prevalence in naturally occurring invertebrate fauna. Comparison of *Campylobacter* isolation rates within differing sample types in a given recreational water site is necessary to address this fundamental question. A number of environmental sources of *Campylobacter* species have been identified that reflect the diversity of potential reservoirs for disease transmission.

2.1.1 Environmental sources of *Campylobacter spp.*

Campylobacter species have been isolated from a vast and variable array of biotic and abiotic sources. Many of these are important reservoirs of *Campylobacter spp.* and play an integral role in their persistence in the environment and possible transmission to members of the public. The following is a summary of the range of environmental sources from which *Campylobacter* has been isolated.

2.1.1.1 Drinking water

Besides food, water (drinking, freshwater, marine) is the single most important vector for transmission of pathogenic *Campylobacter spp.* to humans. Outbreaks of campylobacteriosis while of relatively uncommon occurrence compared to total incidence numbers have been reported locally (Stehr-Green et al., 1991; Bohmer, 1997) as well as globally (Sacks et al., 1986; Duke et al., 1996). Untreated or external contamination of drinking water supplies tend to be responsible for many of these

outbreaks. Contamination is usually attributed to contact with birds, who represent a large source of *Campylobacter spp.* and hence, a persistent threat to drinking water supplies (Sacks et al., 1986; Calder, 1998).

2.1.1.2 Freshwater

The presence of *Campylobacter spp.* in lakes and rivers has been extensively studied (Hill, 1984; Drenchen and Bert, 1994; Bolton et al., 1987; Pianetti et al., 1998; de Boer, 1996; Brennhovd et al., 1992; Arvanitidou et al., 1995). Surface freshwaters become contaminated by faecal inputs from birds, agricultural run-off and leaching from soils (The National Advisory Committee on Microbiological Criteria for Foods, 1995). Contaminated lakes and rivers can therefore pose a public health risk to swimmers and other recreational water users (Medema et al., 1997; de Boer, 1996). Use of freshwater lakes and streams as drinking water for animals (livestock) and birds can serve as a vehicle of transmission between these animals (Stanley et al., 1998). Subsequent faecal inputs to water result in the constant cycling of *Campylobacter spp.* within the aquatic environment.

2.1.1.3 Marine water

Recreational marine waters contaminated with *Campylobacter spp.* have also been reported (Alonso and Alonso, 1993; Obiri-Danso et al., 1999; Levesque et al., 1993). As with drinking water and recreational freshwater, birds (particularly gulls) appear to be the principal source of microbial contamination. Levesque et al. (2000) found Ring-billed gull droppings to contain large numbers of pathogenic bacteria, which contribute to the contamination of marine recreational waters. These pathogenic bacteria (*Campylobacter* being amongst them) can be transmitted to beach swimmers through inadvertent ingestion of such bacterial point sources.

2.1.1.4 Soil

The presence of *Campylobacter spp.* in different soil types has rarely been addressed. Recent studies have isolated (by culture) *Campylobacter* from sand at bathing beaches (Bolton et al., 1999) and from intertidal sediments (Obiri-Danso and Jones, 2000). Detection rates of *Campylobacter* species from sand were as high as 45% (Bolton et al., 1999). Intertidal sediments were consistently positive for *Campylobacter spp.* in

winter months (Obiri-Danso and Jones, 2000). In contrast, *Campylobacter* species could not be found in agricultural soils or stream sediments by culture (Easton, 1996; Jones and Hobbs, 1996). A number of factors such as culture technique used, the soil type and seasonal fluctuations may account for these variable rates. Soil may be an important, but as yet undetermined reservoir for *Campylobacter spp.*

2.1.1.5 Birds

Campylobacter has been shown to be highly prevalent in a number of bird species. Chickens, gulls and waterfowl (ducks, geese, swans) are three important types, with respect to public health. This is largely due to their prevalence within human recreational areas. *Campylobacter spp.* are thought to be normal commensals that form part of avian gut flora (Hasell, 1994). A constant cycle of dissemination of *Campylobacter* into water supplies by birds and reinfection and colonisation from water and between birds exists (Pearson et al., 1993; Hatch, 1996; Glunder et al., 1992; Hussong et al., 1979). Studies addressing the incidence of *Campylobacter* in different bird species have shown highly variable carriage rates. Incidences of 30-97% for gulls (Calder, 1998), 55.5-100% for live chickens (Ridsdale et al., 1998), 41% for pigeons, 40% for rooks, 5-33% for wild geese, 73% for ducks and 81% for Sandhill cranes (Fenlon et al., 1996; Pacha et al., 1988) have been reported. Recently, *Campylobacter spp.* have been isolated from 3% of subantarctic Macaroni penguins in the first apparent report of *Campylobacter* in this region (Broman et al., 2000).

2.1.1.6 Mammals

Campylobacter spp. have been isolated from the intestinal contents of a wide variety of domestic and wild mammals (Rosef et al., 1983). These include pigs, sheep, cattle, horses, dogs and cats (Skirrow, 1990; Rosef et al., 1983; Taylor et al., 1983; The National Advisory Committee on Microbiological Criteria for Foods, 1995, Lindblom et al., 1990; Diker, 1987; Stanley et al., 1998; Hald and Madsen, 1997). Of these animals, the most significant *C. jejuni* isolation rates were from swine (100%) (Rosef et al., 1983), lambs (91.7%) (Stanley et al., 1998), and puppies (29%) (Hald and Madsen, 1997). Gall bladders of sheep have been shown to give a 57.3% isolation rate (Diker, 1987). This indicates that *Campylobacter spp.* are not restricted to the intestine and faecal material of some mammals. Although *Campylobacter spp.* are

carried as part of normal intestinal flora in many mammals, they are sometimes associated with enteric disease in animals such as swine (The National Advisory Committee on Microbiological Criteria for Foods, 1995). *C. jejuni* introduced to infant monkeys have also resulted in disease onset (Russel et al., 1993). Close human contact with carrier animals has been implicated as a source for *Campylobacter spp.* transmission into the human population (Eberhart-Phillips, 1995; Hald and Madsen, 1997).

2.1.1.7 Shellfish

Bacterial and viral gastroenteritis associated with the consumption of raw or undercooked shellfish has a long history (Rippey, 1994). Disease outbreaks have been reported since the late 1800s in the United States (Rippey, 1994). As filter feeding organisms, shellfish are particularly susceptible to microbial contamination from overlying waters. Cockles, mussels, scallops and oysters have all been implicated with disease (Abeyta et al., 1993; Abeyta, 1991). Isolation rates for *Campylobacter spp.* within these shellfish are variable. Isolation rates of between 0-69% have been reported (Wilson and Moore, 1996; Endtz et al. 1997, Bouchriti et al., 1995; Ripabelli et al., 1999; Arumagaswamy and Proudford, 1987). Due to the concentration of *Campylobacter* cells within shellfish tissues, the risk of campylobacteriosis from eating raw shellfish is thought to be substantially greater than ingestion of the surrounding waters (Teunis et al., 1997). To combat this, the process of depuration is used, whereby shellfish are placed into clean seawater tanks to allow for the natural elimination of bacteria from within the animal (Calder, 1998). This process however, is not 100% effective for removal of bacterial pathogens. Wilson and Moore (1999) found 6% of depurated oysters still contained *Campylobacter spp.*

2.1.1.8 Other sources

Campylobacter spp. have been isolated from a number of other sources, which may act as vectors for disease transmission. House flies have been suggested as a possible link for transmission of *Campylobacter spp.* from animals to human food (Khalil et al., 1994; Rosef and Kapperud, 1983). *C. jejuni* carriage rates of 50.7% were reported in flies from a chicken farm (Rosef and Kapperud, 1983).

Retail mushrooms, spinach, lettuce, radish, green onions, parsley and potatoes sold for human consumption have all been shown to harbour *Campylobacter* (Doyle and Schoeni, 1986; Park and Saunders, 1991). Hand-picked Blue crab meat has also been shown to yield *Campylobacter* species (Reinhard et al., 1996). Whether or not this contamination is due to the food handler is uncertain.

2.1.2 Isolation of *Campylobacter spp.* from environmental samples

Isolation of *Campylobacter spp.* from environmental samples implicated in disease outbreaks has been troublesome. Often, this is due to sampling being done weeks after the outbreak coupled with the cellular damage as a result of the organisms constant exposure to harsh environmental conditions (Blaser and Cody, 1986). The link between sporadic cases of campylobacteriosis and environmental water is virtually impossible to demonstrate. The most important factor affecting environmental *Campylobacter* isolation, however, is the sensitivity of the isolation method used. A number of techniques for efficient isolation of the target organism from environmental samples have been trailed, with varying levels of success.

2.1.2.1 Water samples

Early methods of *Campylobacter* isolation from water required the filtration of a water sample through a 0.45μm cellulose acetate membrane followed by placement of the membrane onto blood agar plates containing selective antibiotics (Knill et al., 1978). Membranes are removed from the blood agar after 30min and plates cultured for *Campylobacter* for 24-48h in a process referred to as 'passive filtration'. The 30min membrane placement time is sufficient for motile *Campylobacter* to pass through the membrane with exclusion of most contaminating organisms (Steele and McDermott, 1984). The use of non-selective blood plates was found to increase isolation of antibiotic sensitive *Campylobacter* (Steele and McDermott, 1984; Blaser and Cody, 1986). Subsequent methods replaced the use of blood-based media with selective charcoal based agar–Charcoal Cefoperazone/Cephalothin Deoxycholate Agar (CCDA) (Hutchinson and Bolton, 1984). Both blood and charcoal are thought to decompose toxic oxygen derivatives to promote *Campylobacter* growth (Moran and Upton, 1987). The incubation of post-filtration membranes for 24h instead of 30min has also been used in a modification to passive filtration (Blaser and Cody,

1986). The use of enrichment broths has been shown to substantially increase the yield of thermotolerant *Campylobacters* (Ribeiro and Price, 1984). Membranes from filtered water samples are placed directly into enrichment broth and incubated for 24-48h at 42°C. Broth portions are subsequently spread plated onto selective blood agar plates and incubated for a further 24-48h. The optimum time for subculturing broths was found to be after 48h of broth incubation (Ribeiro and Price, 1984). The simultaneous use of molecular techniques can further increase detection sensitivity.

The antibiotics used for selective broths and solid media are chosen for their ability to inhibit growth of non-target organisms while target organisms are selected for, due to expression of resistance to these agents. The most effective and frequently used antibiotics for isolation of *Campylobacter spp.* include rifampicim (inhibits bacterial RNA polymerase), trimethoprim (interferes with folic acid metabolism), polymixin B (binds to cell membranes and increases permeability to small molecules) and cefoperazone (inhibits formation of cross-links in bacterial peptidoglycan layer) (Mitchell, 1999). Polymixin B acts to inhibit many Gram negative bacteria, trimethoprim primarily inhibits *Proteus spp.* and other Gram negative bacteria, whereas rifampicin and cefoperazone are primary inhibitors of enteric bacteria (especially *Pseudomonas*) and Gram positive bacteria (Corry et al., 1995; Mitchell, 1999). Many of these antibiotics will also show some inhibitory effects on *Campylobacter* species. To combat this, a number of researchers recommend the use of a pre-enrichment period of incubation prior to the addition of antibiotics to broths (Humphrey, 1986; Martin et al., 1996). *Campylobacter* cells present in environmental samples may be sub-lethally damaged due to environmental stresses. These cells show increased sensitivity to antibiotics and inability to grow at the temperature (42°C) used for isolation of thermotolerant *Campylobacter.* Pre-incubation at 37°C in ambient oxygen conditions allow these injured cells to recover before addition of antibiotics. Pre-incubation times vary between 2-24h (Humphrey, 1986; Martin et al., 1996, Calder, 1998). As *Campylobacter* from environmental water sources tend to show the greatest amount of sublethal injury (Mason et al., 1999), the use of a pre-incubation step is integral for efficient *Campylobacter* isolation from broth cultures. In addition, water sampling from a minimum depth of 10cm will reduce the number of UV-light damaged cells in a sample (Armstrong, 1997).

To date, there is no standard broth medium for isolation of *Campylobacter* from water. Consequently, use of a wide variety of selective media coupled with variations in incubation temperature have resulted in significant diversity in the range and proportions of *Campylobacter spp.* recovered from environmental specimens (Lastovica and Frost, 1996; Ng et al., 1988; Gun-Munro et al., 1987; Abeyta et al., 1996; Tran, 1998; Lopez et al., 1998; Engberg et al., 2000). 'Exeter' enrichment broth has been shown to be particularly effective for isolation of *Campylobacter* species from water (Humphrey et al., 1995; Martin et al., 1996; Mason et al., 1996). Exeter enrichment broth contains a number of components to protect *Campylobacter spp.* from the toxic effect of oxygen derivatives. These include defibrinated sheep's blood and ferrous sulphate, sodium metabisulphite, sodium pyruvate (FBP) supplement (Corry et al., 1995). Use of a pre-incubation step coupled with Exeter broth isolation has been shown to optimise *Campylobacter* recovery from water (Martin et al., 1996). However, the opposite is true for *Campylobacter* isolation from chicken washes where a pre-incubation step was not required (Martin et al., 1996).

2.1.2.2 Aquatic invertebrate samples

The isolation of *Campylobacter* species from aquatic invertebrate samples generally requires the de-shelling of the animal followed by homogenisation of flesh in a blender. Homogenisation is done either in the absence of a diluent or in peptone water (Bouchriti et al., 1995; Endtz et al., 1997). Aliquots of the flesh homogenate are placed into selective enrichment broths such as 'Exeter' broth and cultured for isolation. For shellfish, the use of the enzyme Oxyrase added to specialised broth has been shown to be effective for isolation of *Campylobacter spp.* (Abeyta et al., 1997).

2.1.3 The Avon-Heathcote Estuary

The Avon-Heathcote Estuary is a large natural ecosystem surrounded by parts of Christchurch city (Royds Garden, 1993). It is situated approximately 6.5km southeast of central Christchurch (Dall, 1985; Robb, 1981). The Estuary is roughly the shape of an equilateral triangle containing 716ha of tidal mudflats with an area of approximately eight square kilometres (Knox and Kilner, 1973). Two rivers flow into the estuary, the Avon River enters at the northern corner and the Heathcote River

from the western corner (Robb, 1974). The eastern margin of the estuary is bound by a narrow sand spit, which separates the estuary from the South Pacific Ocean (Robb, 1981). It is estimated that approximately 8.5 million cubic metres of seawater enters the estuary at high tide (Royds Garden, 1993). The Avon and Heathcote Rivers contribute to the majority of freshwater entering the estuary with 53% contributed by the Avon River and 18% by the Heathcote River (Robb, 1981). The remaining 29% of freshwater entering the estuary is discharged from the Bromley oxidation ponds and rainfall (Knox and Kilner, 1973).

2.1.3.1 The Bromley oxidation ponds

The Bromley oxidation ponds are a prominent feature of the estuary. Six ponds treat and discharge effluent into the estuary. Daily discharge of effluent into the estuary is approximately 134,000 cubic metres (Royds Garden, 1993). It is estimated that 80-90% of this discharge is removed to the open ocean (Royds Garden, 1993). Bird numbers within the estuary have significantly increased due to the oxidation ponds providing enrichment for the food chain (Crossland, 1993). The injection of algae and protozoa from the oxidation ponds has also increased the biomass of filter feeders (cockles, mussels, crabs) that consume these organisms (Stephenson, 1981; Royds Garden, 1993).

2.1.3.2 The Avon River

The Avon River flows through the northwestern suburbs of inner Christchurch (Robb, 1981). It is spring-fed, 26km in length and is served by a number of tributaries including natural streams, creeks and two large man-made drains. It is estimated that a low flow of 2.7 m^3/s exists at the river's point of entry to the estuary (Robb, 1981). The total area of the river catchment is 84.3 square kilometres and rises to 30m above sea level (Royds Garden, 1993).

2.1.3.3 The Heathcote River

The Heathcote River is spring-fed, slow-flowing, and meandering. It flows through the southern suburbs of Christchurch including much rural land and the northern slopes of the Port Hills (Royds Garden, 1993). The Heathcote River is 25km in length with an estimated low flow of 0.89 m^3/s at its point of entry to the estuary (Robb,

1981). The river catchment area is much larger and higher than the Avon, at 103.4 square kilometres (Royds Garden, 1993).

2.1.3.4 Cockles

The cockle (*Chione stutchburyi*) is the most abundant bivalve in the estuary (Knox and Kilner, 1973). Its distribution is limited by a number of factors, the most important of which is salinity (Marsden and Pilkington, 1995). The greatest abundance and largest sized cockles are found near the Beechville road jetty on the southern border of the estuary. The salinity of the water at this region is 30ppt. Cockles are less abundant and much smaller in size at regions of the estuary with water of a lower salinity (Marsden and Pilkington, 1995). Salinities of less than 18ppt substantially reduce feeding of cockles (Wong and Thomson, 1992). Cockles are indiscriminate filter feeders and the nature of material ingested reflects the environmental characteristics of the area occupied (Wong and Thomson, 1992). Therefore, cockles may make suitable candidate bio-indicators by accumulating any *Campylobacter* present in the overlying waters to detectable levels.

2.1.3.5 Mudflat snails

The tidal mudflat snail (*Amphibola crenata*) is the only marine pulmonate gastropod that retains its operculum (Griffin and Thomson, 1992). It is tolerant to a wide range of salinities and as a result, has a vast distribution in Avon-Heathcote Estuary. Its abundance is particularly high along the northeastern border of the estuary where the salinity of the water is 20ppt (Marsden and Pilkington, 1995). At low tide, these snails are completely uncovered (Griffin and Thomson, 1992). The mudflat snail is primarily a grazer, feeding on algae and protozoa on sediments. Snails may accumulate *Campylobacter spp.* that sink to the sediments from the water and thus, could also be a candidate bio-indicator for *Campylobacter* contamination of estuarine waters.

2.1.3.6 Birdlife

The Avon-Heathcote Estuary supports a large and varied bird population (Royds Garden, 1993). Between 1980 and 1992, 102 species of birds were recorded in the estuary (Crossland, 1993). The average number of wetland birds using the estuary,

the oxidation ponds and their margins has been estimated to be between 15,000 and 22,000 with upwards of 36,000 birds in late summer-autumn. Gulls, terns, shags, pukeko, swallows, herons, spoonbills, kingfishers and waterfowl (ducks, geese, swans) are the predominant bird types present within the estuary (Crossland, 1993; Hawke, 1998).

Birdlife on the Avon and Heathcote Rivers undergoes numerous changes due to bird behavioural patterns and changing environmental conditions (Royds Garden, 1993). Black-billed gulls are the dominant riverside gull species with increasing numbers of southern black-backed and red-billed gulls being encountered. Paradise ducks, grey ducks and shoveler ducks are also common along the rivers (Royds Garden, 1993).

2.1.3.8 Microbial inputs to the Avon-Heathcote Rivers and estuary

Microbial contamination of the estuary, oxidation ponds and the Avon and Heathcote Rivers is attributed in part to the large population of birdlife, particularly waterfowl (Robb, 1974). However, the contribution of the effluent discharge from the Bromley oxidation ponds into the estuary is estimated to be 94% of bacterial loading in dry weather (Royds Garden, 1993). During periods of rainfall, significant bacterial loading enters the estuary from the Avon and Heathcote Rivers.

Bacterial loading into the Avon and Heathcote Rivers is also significant. Two major sources of bacterial contamination are recognised. Firstly, both rivers receive stormwater run-off from drains that empty into the rivers (Robb, 1981). The second major source of bacterial contamination is from faecal inputs from the large bird populations present along each river. Seagulls and ducks are assumed to be the largest contributors of bird-based contamination. As previously stated, birds are a significant reservoir of *Campylobacter spp.* Therefore, the presence of at least point sources of *Campylobacter* would be expected in both rivers.

2.1.3.9 Shellfish quality

Bacteria reaching the estuary in wastewater are readily accumulated by filter feeding shellfish such as cockles (Royds Garden, 1993). Consequently, this uptake of pathogens becomes a threat to public health if shellfish are used for human

consumption before the shellfish eliminates the microbe through digestion. Faecal coliforms have been isolated from both the Avon and Heathcote Rivers at their point of entry into the estuary. In numerous instances, this is considerably in excess of the current guidelines of 14 MPN faecal coliforms per 100ml of water in shellfish-gathering waters (McBride and Cooper, 1992; Robb, 1981). This, coupled with the bacterial inputs from the oxidation ponds would make cockles generally unsafe to eat. Three major studies assessing shellfish quality between 1977 and 1992 have confirmed this notion (Royds Garden, 1993). In these studies, a large number of samples exceeded the shellfish standard of 'no more than 2 of 5 samples should contain faecal coliforms in excess of 230 per 100g of wet weight (flesh and shellwater) (Royds Garden, 1993; McBride and Cooper, 1992).

2.1.4 The Groynes recreational area

The Groynes recreational area is situated in the southern part of the Waimakariri catchment, situated north of central Christchurch. It is used frequently as a picnicking area and for swimming, fishing, and canoeing (Armstrong, 1997). The Groynes has a large bird population, particularly ducks, which may contribute to the *Campylobacter* contamination of water. A site map and overview of water quality at The Groynes is detailed by Armstrong (1997).

The high levels of microbial inputs into the Avon-Heathcote rivers and estuary system and The Groynes recreational area suggest that these waters may harbour pathogenic *Campylobacter spp.* that could pose a threat to public health.

2.1.5 Objectives for environmental *Campylobacter spp.* isolation

The primary focus of this chapter is to test for the presence of *Campylobacter spp.* from a range of environmental water sites and invertebrate types from the Avon-Heathcote estuary. The specific aims are to:

1. Evaluate the most sensitive and appropriate method for environmental isolation of *Campylobacter spp.* from recreational water

2. Take water samples from a range of recreational water types in and around the Avon-Heathcote estuary, in order to establish whether or not these water sites are a reservoir for *Campylobacter* species

3. Test a range of aquatic invertebrates for the presence of *Campylobacter spp.* from recreational water sites previously established as reservoirs and non-reservoirs of *Campylobacter spp.* from aim # 2 above

4. Place into the water, a potential biological indicator (the freshwater mussel) not common to a water supply, to assess bio-accumulation capabilities

5. Make comparisons between isolation rates from the invertebrates and the surrounding water to assess suitability as a bio-indicator in the natural environment.

2.2 Materials and Methods

2.2.1 Bacterial strains

All bacterial strains used for the duration of this thesis are listed in Table 2.1.

Table 2.1 Bacterial strains used during this study

Collection No.	Isolate code	Description	Source/Reference
KLC4235	762525	*C. jejuni*, Human faeces	Medlab South Ltd, Canterbury
KLC4356	F38011	*C. jejuni*, Human faeces	M. E. Konkel, Washington, USA
KLC4357	M275	*C. jejuni*, Human faeces	M. E. Konkel, Washington, USA
KLC4133	MS961029	*C. jejuni*, Ashburton water outbreak	Medlab South Ltd, Canterbury
KLC4219	732850	*C. jejuni*, Human faeces	Medlab South Ltd, Canterbury
KLC4220	726759	*C. jejuni*, Human faeces	Medlab South Ltd, Canterbury
KLC4221	740081	*C. jejuni*, Human faeces	Medlab South Ltd, Canterbury
KLC4222	736624	*C. jejuni*, Human faeces	Medlab South Ltd, Canterbury
KLC4223	739813	*C. jejuni*, Human faeces	Medlab South Ltd, Canterbury
KLC4224	736927	*C. jejuni*, Human faeces	Medlab South Ltd, Canterbury
KLC4225	739822	*C. jejuni*, Human faeces	Medlab South Ltd, Canterbury
KLC4227	726756	*C. jejuni*, Human faeces	Medlab South Ltd, Canterbury
KLC4228	740102	*C. jejuni*, Human faeces	Medlab South Ltd, Canterbury
KLC4229	722307	*C. jejuni*, Human faeces	Medlab South Ltd, Canterbury
KLC4230	733034	*C. jejuni*, Human faeces	Medlab South Ltd, Canterbury
KLC4231	760888	*C. jejuni*, Human faeces	Medlab South Ltd, Canterbury
KLC4232	760624	*C. jejuni*, Human faeces	Medlab South Ltd, Canterbury
KLC4233	764023	*C. jejuni*, Human faeces	Medlab South Ltd, Canterbury
KLC4234	762234	*C. jejuni*, Human faeces	Medlab South Ltd, Canterbury
KLC4237	770122	*C. jejuni*, Human faeces	Medlab South Ltd, Canterbury
KLC4238	780314	*C. jejuni*, Human faeces	Medlab South Ltd, Canterbury
KLC4239	770928	*C. jejuni*, Human faeces	Medlab South Ltd, Canterbury
KLC4240	768982	*C. jejuni*, Human faeces	Medlab South Ltd, Canterbury
KLC4241	773910	*C. jejuni*, Human faeces	Medlab South Ltd, Canterbury
KLC4242	768407	*C. jejuni*, Human faeces	Medlab South Ltd, Canterbury
KLC4243	770608	*C. jejuni*, Human faeces	Medlab South Ltd, Canterbury
KLC4244	776985	*C. jejuni*, Human faeces	Medlab South Ltd, Canterbury
KLC4245	780015	*C. jejuni*, Human faeces	Medlab South Ltd, Canterbury
KLC4246	786483	*C. jejuni*, Human faeces	Medlab South Ltd, Canterbury
KLC4247	769100	*C. jejuni*, Human faeces	Medlab South Ltd, Canterbury
KLC4249	776736	*C. jejuni*, Human faeces	Medlab South Ltd, Canterbury
KLC4250	787321	*C. jejuni*, Human faeces	Medlab South Ltd, Canterbury
KLC4270	739583	*C. coli*, Human faeces	Medlab South Ltd, Canterbury
KLC4271	722841	*C. coli*, Human faeces	Medlab South Ltd, Canterbury
KLC4272	736268	*C. coli*, Human faeces	Medlab South Ltd, Canterbury
KLC4273	722434	*C. coli*, Human faeces	Medlab South Ltd, Canterbury
KLC4274	736592	*C. coli*, Human faeces	Medlab South Ltd, Canterbury
KLC4275	739584	*C. coli*, Human faeces	Medlab South Ltd, Canterbury
KLC4276	722753	*C. coli*, Human faeces	Medlab South Ltd, Canterbury
KLC4277	WA30	*C. jejuni*, Lesters Stream, Canterbury	ESR, Canterbury. NZ Culture Collection
KLC4278	WA29	*C. jejuni*, Waimakariri River, Canterbury	ESR, Canterbury. NZ Culture Collection
KLC4279	WA39	*C. jejuni*, Bromley Pond, Canterbury	ESR, Canterbury. NZ Culture Collection
KLC4280	WA33	*C. jejuni*, Coes Ford, Canterbury	ESR, Canterbury. NZ Culture Collection
KLC4281	WA31	*C. coli*, Lesters Stream, Canterbury	ESR, Canterbury. NZ Culture Collection

Table 2.1 Continued...

Collection No.	Isolate code	Description	Source/Reference
KLC4282	WA27	*C. coli*, Bromley Pond, Canterbury	ESR, Canterbury. NZ Culture Collection
KLC4283	WA38	*C. coli*, Bromley Pond, Canterbury	ESR, Canterbury. NZ Culture Collection
KLC4284	WA36	*C. coli*, Bromley Pond, Canterbury	ESR, Canterbury. NZ Culture Collection
KLC4297	HS023	*C. jejuni*, Hamilton	KSC, Wellington, NZ
KLC4300	HS036	*C. jejuni*, Palmerston North	KSC, Wellington, NZ
KLC4315	HS019	*C. jejuni*, Human faeces	KSC, Wellington, NZ
KLC4325	AC001	*C. jejuni*, Seagull, Avon-Heathcote Estuary	Calder, 1998
KLC4326	AC002	*C. lari*, Seagull Avon-Heathcote Estuary	Calder, 1998
KLC4327	AC003	*C. jejuni*, Mallard Duck, Lake Clearwater	Calder, 1998
KLC4328	AC008	*C. jejuni*, Mallard Duck, Lake Clearwater	Calder, 1998
KLC4329	AC011	*C. jejuni*, Mallard Duck, Lake Clearwater	Calder, 1998
KLC4330	AC013	*C. jejuni*, Mallard Duck, Lake Clearwater	Calder, 1998
KLC4331	AC017	*C. jejuni*, Mallard Duck, Lake Clearwater	Calder, 1998
KLC4332	AC021	*C. jejuni*, Mallard Duck, Lake Clearwater	Calder, 1998
KLC4333	AC025	*C. jejuni*, Mallard Duck, Lake Clearwater	Calder, 1998
KLC4334	AC029	*C. jejuni*, Mallard Duck, Lake Clearwater	Calder, 1998
KLC4335	AC032	*C. jejuni*, Mallard Duck, Lake Clearwater	Calder, 1998
KLC4336	AC034	*C. jejuni*, Mallard Duck, Lake Clearwater	Calder, 1998
KLC4337	AC037	*C. jejuni*, Mallard Duck, Lake Clearwater	Calder, 1998
KLC4338	AC039	*C. jejuni*, Mallard Duck, Lake Clearwater	Calder, 1998
KLC4340	AC051	*C. jejuni*, Mallard Duck, Lake Clearwater	Calder, 1998
KLC4341	AC055	*C. jejuni*, Canada Goose, Parnassus	Calder, 1998
KLC4342	AC056	*C. jejuni*, Canada Goose, Parnassus	Calder, 1998
KLC4343	AC058	*C. jejuni*, Canada Goose, Parnassus	Calder, 1998
KLC4344	AC062	*C. jejuni*, Canada Goose, Parnassus	Calder, 1998
KLC4345	AC064	*C. jejuni*, Canada Goose, Parnassus	Calder, 1998
KLC4346	AC066	*C. jejuni*, Canada Goose, Parnassus	Calder, 1998
KLC4347	AC068	*C. jejuni*, Canada Goose, Parnassus	Calder, 1998
KLC4348	AC071	*C. jejuni*, Canada Goose, Parnassus	Calder, 1998
KLC4349	AC073	*C. jejuni*, Rakaia River Water	Calder, 1998
KLC4350	AC079	*C. jejuni*, Estuary Water	Calder, 1998
KLC4351	AC081	*C. lari*, Seagull, Avon-Heathcote Estuary	Calder, 1998
KLC4352	AC083	*C. lari*, Seagull, Avon-Heathcote Estuary	Calder, 1998
KLC4353	AC085	*C. jejuni*, Seagull, Avon-Heathcote Estuary	Calder, 1998
KLC4354	AC087	*C. lari*, Seagull, Avon-Heathcote Estuary	Calder, 1998
KLC4355	AC089	*C. lari*, Seagull, Avon- Heathcote Estuary	Calder, 1998
KLC5000	ZH614W	*C. jejuni*, Human faeces	Canterbury Health Laboratories

Table 2.1 Continued...

Collection No.	Isolate code	Description	Source/Reference
KLC5001	ZI814K	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5003	LT973B	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5004	LS785M	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5005	LS897W	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5006	DO291I	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5007	DO332T	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5008	DN031T	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5009	CI115W	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5010	BB193H	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5012	XT318V	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5013	RC317T	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5014	HW961T	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5015	CU130P	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5016	CM123F	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5017	BV3740	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5018	BN499U	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5019	BM271M	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5020	XM653F	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5021	XX158F	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5022	RC703J	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5023	MZ347T	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5024	PA268R	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5025	LG430I	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5026	HI881O	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5027	YU298M	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5028	YN438B	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5029	LC667G	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5030	YP567F	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5031	YO808T	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5032	XV898P	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5033	RD177L	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5034	QY139W	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5035	IG022P	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5036	ZB375B	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5037	LB445E	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5038	RC167B	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5039	ZP028D	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5040	ZB632B	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5049	SO52	*C. jejuni*, Human Blood	Canterbury Health Laboratories
KLC5050	R923	*C. jejuni*, Human Blood	Canterbury Health Laboratories
KLC5081	QA598G	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5082	PN728G	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5083	PD850L	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5084	ZN244B	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5085	PH844R	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5086	PH845S	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5087	PH790A	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5088	MS179P	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5089	KV955A	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5090	ZJ638R	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5091	FZ917J	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5092	YJ936T	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5093	YG023Q	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5094	YG018S	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5095	MC591P	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5096	MC715F	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5097	MD672W	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5098	ZI975L	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5099	ZH606P	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5100	ZH6050	*C. jejuni*, Human faeces	Canterbury Health Laboratories

Table 2.1 Continued...

Collection No.	Isolate code	Description	Source/Reference
KLC5101	MM539G	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5102	LG794W	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5103	DO399V	*C. jejuni*, Human faeces	Canterbury Health Laboratories
KLC5104	ZH604N	*C. jejuni*, Human faeces	Canterbury Health Laboratories
	CJS78	*C. jejuni*, Sheep	Massey University, Palmerston North
	CJS79	*C. jejuni*, Sheep	Massey University, Palmerston North
	CJS83	*C. jejuni*, Sheep	Massey University, Palmerston North
	CJS84	*C. jejuni*, Sheep	Massey University, Palmerston North
	CJS85	*C. jejuni*, Sheep	Massey University, Palmerston North
	CJS86	*C. jejuni*, Sheep	Massey University, Palmerston North
	CJS87	*C. jejuni*, Sheep	Massey University, Palmerston North
	CJS88	*C. jejuni*, Sheep	Massey University, Palmerston North
	CJS89	*C. jejuni*, Sheep	Massey University, Palmerston North
	CJS90	*C. jejuni*, Sheep	Massey University, Palmerston North
	SS0124	*C. lari*	ESR, Canterbury, NZ culture collection
	SS0127	*C. coli*	ESR, Canterbury, NZ culture collection
	NZRM3242	*C. jejuni*, ATCC33291, Human faeces	Laboratory Culture Collection
	NZRM698	*Bacillus subtilus*	Laboratory Culture Collection
	NZRM3178	*Enterococcus faecalis*	Laboratory Culture Collection
	NZRM2749	*E. coli*, NCTC11560	Laboratory Culture Collection
	NZRM916	*E. coli*, ATCC25922, NCTC12241	Laboratory Culture Collection
	NZRM3441	*E. coli*, O157:H7	Laboratory Culture Collection
	NZRM3184	*Haemophilus influenzae*, ATCC49247	Laboratory Culture Collection
	NZRM3245	*Haemophilus influenzae*, ATCC10211	Laboratory Culture Collection
	NZRM3315	*Haemophilus influenzae*, ATCC49766	Laboratory Culture Collection
	NZRM65	*Morganella morganii*, ATCC25830, NCTC235	Laboratory Culture Collection
	NZRM155	*Neisseria meningitidis*, ATCC13090	Laboratory Culture Collection
	NZRM1036	*Neisseria gonorrhoeae*, ATCC49226	Laboratory Culture Collection
	NZRM2590	*Neisseria lactamica*, ATCC23970	Laboratory Culture Collection
	NZRM1011	*Pastuerella volantium*	Laboratory Culture Collection
	NZRM805	*Plesiomonas shigelloides*	Laboratory Culture Collection
	NZRM918	*Pseudomonas aeuriginosa*, ATCC27853	Laboratory Culture Collection
	NZRM2243	*Staphylococcus aureus*, ATCC29213	Laboratory Culture Collection
	NZRM87	*Staphylococcus aureus*, ATCC9144, NCTC6571	Laboratory Culture Collection
	NZRM103	*Staphylococcus aureus*	Laboratory Culture Collection
	NZRM94	*Streptococcus dysgalactiae subsp. Equisimilis*, ATCC35666, NCTC8543	Laboratory Culture Collection

Table 2.1 Continued...

Collection No.	Isolate code	Description	Source/Reference
	NZRM3399	*Streptococcus pneumoniae*, ATCC49619	Laboratory Culture Collection
	NZRM3201	*Streptococcus pneumoniae*, ATCC6305	Laboratory Culture Collection
	NZRM2723	*Streptococcus pyogenes*, ATCC19615	Laboratory Culture Collection
	NZRM3250	*Streptococcus spp.*, ATCC12386	Laboratory Culture Collection
	NZRM95	*Streptococcus spp.*, NCTC9603	Laboratory Culture Collection
	NZRM2603	*Yersinia enterocolitica*	Laboratory Culture Collection
	NZRM3476	*Shigella flexneri*, ATCC12022, NCTC12698	Laboratory Culture Collection
	NZRM3205	*Aeromonas hydrophila*, ATCC7965, NCTC7812	Laboratory Culture Collection
	NZRM816	*Arcanobacterium haemolyticum*, NCTC9697	Laboratory Culture Collection
	NZRM2565	*Branhamella catarrhalis*, ATCC25238, NCTC11020	Laboratory Culture Collection
	NZRM3226	*Salmonella typhimurium*, ATCC14028, NCTC12023	Laboratory Culture Collection
	NZRM23	*Corynebacterium diptheriae*, ATCC19409, NCTC3984	Laboratory Culture Collection
	NZRM820	*Vibrio parahaemolyticus*, ATCC43996, NCTC10884	Laboratory Culture Collection

2.2.2 Buffers and media

Media used in this study were prepared as described in appendix I. General buffers and solutions used were prepared as described in appendix II.

2.2.3 Antibiotics and supplements

Antibiotics and supplements were added to media to provide a selection pressure or enhance growth of the target organism. Concentrations of antibiotics and supplements used are listed in Table 2.2. Compositions of supplements are described in appendix I.

Table 2.2 Antibiotics and supplements added to selective media

Antibiotic/Supplement	Abbreviation	Final Concentration
Sodium cefoperazone	Cef	3.2mg/L
Tetracycline hydrochloride	Tet	15mg/L
Polymixin B sulphate[A]	PmB	5000iu/L
Trimethoprim[A]	Tmp	10mg/L
Rifampicin[A]	Rif	10mg/L
Cyclohexamide[A]	Chx	100mg/L
Solution A[B]	Sol. A	10ml/L

A: These four antibiotics are combined to form supplement B for Exeter broths
B: See appendix II for composition of solution A

2.2.4 Sterilisation techniques

2.2.4.1 Autoclaving

All media, glassware and equipment were autoclaved at 121°C at a pressure of 103.4 kPa for 20min.

2.2.4.2 Filter sterilisation

Antibiotics and reagents unable to be autoclaved were filter sterilised by passing through a 0.22μm pore size cellulose nitrate filter (Sartorious) into a sterile vessel.

2.2.4.3 Implements

All implements (eg., scalpel, shucking knife, inoculating loops) used were autoclaved where possible. When autoclaving was not possible, implements were surface sterilised with 70% ethanol in between manipulations.

2.2.5 Standard culture conditions for *Campylobacter* species

Campylobacter strains were cultured by streak isolation on Oxoid *Campylobacter* blood-free selective agar base (modified CCDA-Preston) containing 3.2 mg/L of cefoperazone (Cef). Plates containing cells were incubated for 24-48h at 37°C under a reduced oxygen atmosphere (10% CO_2, 6% O_2, 84% N_2) in a water-jacketed CO_2 incubator (NuAire).

2.2.6 Long term storage of *Campylobacter* isolates

For long term storage, bacterial growth from a CCDA plate was harvested using a 6:1 mixture of brain heart infusion broth (BHI) (Merck) to glycerol (BDH) and a glass spreader. The BHI/glycerol solution (2.5ml) was added to plates and bacteria dislodged using an ethanol-sterilised glass spreader. The resultant suspension was aseptically transferred to sterile 1.8ml Nunc cryotubes containing six 2.5mm diameter glass beads (BDH). Cryotubes were stored at -80°C until required. *Campylobacter* cells were recovered from -80°C stocks by rotating a glass bead over the surface of a CCDA plate from a thawed cryotube.

2.2.7 Enumeration of *C. jejuni* cellular growth

Cellular growth from a 24-48h pure culture of *C. jejuni* was harvested using 5ml phosphate buffered saline (PBS) solution (appendix II) and a sterile glass spreader. The cell suspension was aseptically transferred to a sterile universal bottle and referred to as the 'stock solution'. Ten-fold serial dilutions of the stock solution were prepared by transferring 1ml of solution to 9ml of sterile PBS. Aliquots of 100µl were spread plated onto CCDA plates in triplicate. Plates were allowed to dry for 15min in an Email air handling biological safety cabinet [class II] and incubated for 24-48h under microaerophilic conditions. Following incubation, bacterial colonies were counted. Atypical *Campylobacter* colonies were checked by Gram stain. Plates yielding colony counts of between 20-200 were averaged from each triplicate set in order to quantify *C. jejuni* colony forming units (cfu/ml) in stock solution.

2.2.8 Detection of *C. jejuni* from seeded samples

In order to optimise environmental isolation of *Campylobacter* species, it was necessary to select a sufficiently sensitive isolation method to detect potentially low numbers of *Campylobacter* present in environmental water. The sensitivity of two isolation methods (passive filtration and enrichment) were evaluated.

2.2.8.1 Detection limits of passive filtration

C. jejuni F38011 was used as the test strain for passive filtration experiments. Aliquots of 249ml of sterile distilled water (dH_2O) were artificially seeded with 1ml of ten-fold serial dilutions of *C. jejuni* F38011. Seeded water samples were immediately placed into sterile filter holders (Sartorious, Germany) and passed through a cellulose nitrate 0.45µm pore size filter with the aid of a vacuum. Filtrates were discarded and filter membranes were placed face up on CCDA plates and incubated in a reduced O_2 atmosphere for 30min at 37°C. Filters were subsequently removed and plates incubated for a further 48h. Passive filtration experiments were performed in duplicate to ensure reproducibility of results.

2.2.8.2 Detection limits of C. jejuni enrichments

In order to determine the minimum number of cells required to be introduced to enrichment broths for detection by culture, Exeter enrichment broths were seeded

with known quantities of *C. jejuni*. Exeter enrichment broth minus supplement B was prepared as described in appendix I. *C. jejuni* KLC4235 (tet^R) was chosen as the test strain for these detection experiments. Aliquots of 99ml of Exeter enrichment broth were aseptically placed into sterile whirl-pac bags (Labplas) and artificially seeded with 1ml of ten-fold serial dilutions of the *C. jejuni* test strain. Whirl-pac bags containing seeded broth were pre-incubated for 2h at 37°C under atmospheric O_2 conditions. Following pre-incubation, supplement B, Cef, and tetracycline (Tet) (see table 2.2 for concentrations) were added and broths incubated for a further 24-48h in a reduced O_2 atmosphere at 37°C. Following incubation, 100ul aliquots of broth were spread onto CCDA using a glass spreader and plates incubated for 48h in microaerophilic conditions.

2.2.9 Isolation of *Campylobacter* species from recreational water

Water samples were taken from in and around the Avon-Heathcote estuary system in order to determine whether these waters harbour culturable *Campylobacter* species.

2.2.9.1 Sampling sites

Five major sampling sites were used for environmental sampling. The location of each of these sites are indicated in Figure 2.1.

2.2.9.1.1 Avon River site

This site spans a 1km section of the Avon River marked by a walk bridge at either end. The site is a particularly busy region of the river, which begins at the Christchurch Public Hospital and ends within the Christchurch Botanic Gardens. This area was chosen primarily because of its daily use for recreational canoeing.

2.2.9.1.2 Heathcote River site

The Heathcote River site also encompasses a 1km section marked by a walk bridge at each end. This site is situated along Aynsley Terrace in a suburban region of Christchurch. It was chosen as a representative of the second river flowing into the estuary.

Figure 2.1: Map of the Christchurch Avon-Heathcote Estuary and rivers showing the major sampling sites used for this study. A= Estuary South Brighton site; B= Beechville Rd jetty site; C= Sumner-Cave Rock site; D= Avon River site and E= Heathcote River site.

2.2.9.1.3 Beechville Rd Jetty site

The Beechville Rd jetty is situated within the estuary on the south-eastern side. At high tide, this region is used for kayaking and boating. This site was chosen due to the frequent distribution and high abundance of particularly large cockles (*Chione stutchburyi*).

2.2.9.1.4 Sumner-Cave Rock site

This site is a popular recreational beach representing the eastern shore of the Pacific Ocean, which the estuary empties into. This site was chosen because it has an abundance of shellfish beds containing the blue-shelled mussel (*Mytilus edulis*) and is a common swimming area.

2.2.9.1.5 Estuary South Brighton site

This site is situated within the estuary on the north-eastern edge. The site was chosen because it contains a high abundance of estuarine mudflat snails (*Amphibola crenata*).

2.2.9.2 Water sampling

Water samples were collected in 1L sterile glass Schott bottles. Sampling was done >10cm below the surface of the water to optimise isolation of non-UV damaged cells. Care was taken to avoid mixing of sediments present beneath the water at point of sampling. Schott bottles were filled to maximum capacity excluding air where possible. Samples were held on ice during transportation to the laboratory and were processed immediately.

2.2.9.3 Processing of water samples

A 0.45μm pore size cellulose nitrate filter was placed onto a sterile filter holder and covered with 2-3 layers of sterile muslin cloth. The muslin cloth was used for pre-filtration to prevent any large particulate debris from clogging the filter. Each 1L water sample was subsequently passed through the membrane filter using a vacuum aspirator. Filtrates and muslin cloth were discarded and membrane filters aseptically placed into sterile whirl-pac bags containing 100ml of Exeter enrichment broth excluding antibiotic supplement B.

2.2.9.4 Enrichment conditions

Whirl-pac bags were pre-incubated for 2h at 37°C under atmospheric O_2 conditions. Pre-incubation allows any damaged *Campylobacter* cells to recover before addition of selective antibiotics. Broths were incubated for 24-48h under a reduced O_2 atmosphere at 37°C after addition of antibiotic supplement B. Aliquots of 100µl and 50µl of broth were spread plated onto CCDA in duplicate to optimise recovery of environmental *Campylobacter spp.* Plates were incubated for 24-48 hours in microaerophilic conditions.

2.2.10 Isolation of *Campylobacter* species from aquatic invertebrates

Aquatic invertebrates common to the Avon-Heathcote Rivers and estuary system were tested for the presence of *Campylobacter spp.* Three major invertebrate types were examined (Figure 2.2).

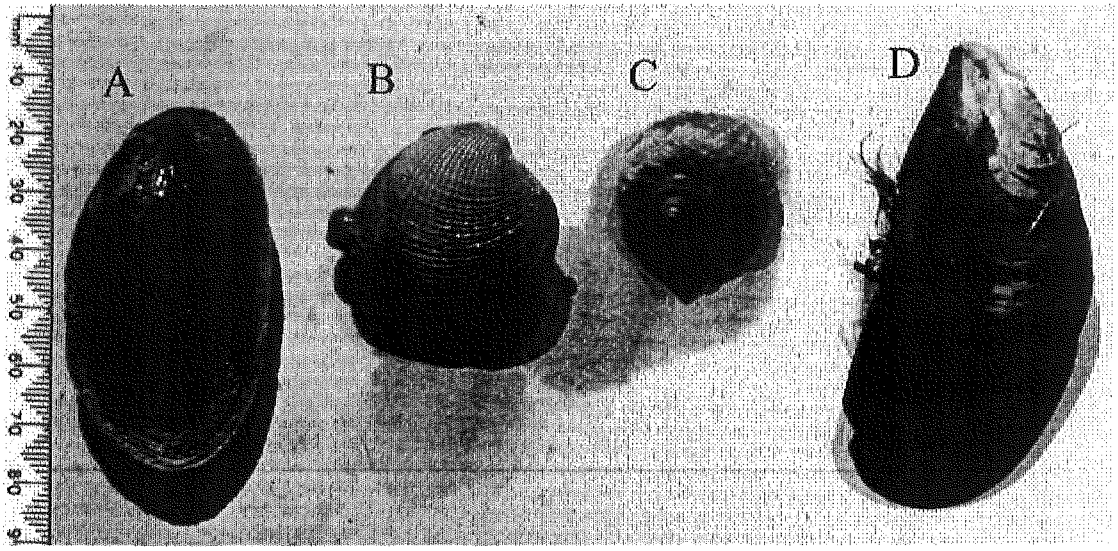

Figure 2.2: Aquatic invertebrates sampled during this study. A= Freshwater mussel (*Hydridella menziesi depauperta*); B= Cockle (*Chione stutchburyi*); C= Estuarine snail (*Amphibola crenata*) and D= Marine Blue-shelled mussel (*Mytilus edulis*).

2.2.10.1 Cockles

Cockles (*Chione stutchburyi)* were collected from the Beechville Rd jetty site. Sampling was done during low tide when cockles were easily accessible. Four individual cockles were collected in sterile whirl-pac bags containing a small amount of the surrounding water and transported back to the laboratory on ice. Each bag

containing 4 cockles was referred to as 'one sample' and was processed accordingly. Animals were 4cm in size with an average flesh weight (including shellwater) of 8.36g. Cockles were processed immediately upon return to the laboratory. A sample of water was always taken in the vicinity of the cockle collection area.

2.2.10.2 Estuarine snails

Estuarine snails (*Amphibola crenata)* were collected from the South Brighton estuary site. Sampling was done at mid-tide when animals were completely submerged in the surrounding water but were still accessible. Ten small or five large snails were collected in sterile whirl-pac bags containing a small amount of estuarine water (ie., one sample) and transported back to the laboratory on ice. Large snails were 1.8cm in size and had an average weight of 2.73g whereas small snails were 0.7cm in size and had an average weight of 1.3g.

2.2.10.3 Marine mussels

Blue-shelled mussels (*Mytilus edulis*) were collected from the Sumner cave rock site. Mussel sampling was done in an identical manner to that of cockles (see section 2.2.10.1). Only mussels completely submerged in water at low tide (at the bottom of the shellfish bedrock) were collected. A 1L water sample from the Pacific Ocean was always included with mussel samples. Mussels averaged 7.4cm in size and had an average flesh weight of 15.4g including shellwater.

2.2.10.4 Processing of invertebrate samples

Mussels and cockles were shucked using sterile implements (see section 2.2.4.3). Flesh and shellwater from each sample type of four shellfish were blended in a sterile Waring blender with 100ml of Exeter enrichment broth (excluding supplement B) until a uniform consistency was achieved. The shellfish flesh/broth mixture was poured into a sterile whirl-pac bag and enriched as described in section 2.2.9.4.

Snails were blended whole in a sterile Waring blender as described above. Each animal had to be placed into the blender individually to prevent snail shells from blocking the blender blade. Snails were enriched in identical conditions to that of shellfish.

2.2.11 Purification of Environmental *Campylobacter spp.*

Moist, flat, grey, spreading colonies with a metallic sheen present on post-incubation CCDA plates were re-streaked onto fresh CCDA plates using a sterile wire inoculating loop. The streak isolation technique was used to obtain single colonies. Individual colonies were passaged 3-5 times on CCDA to ensure purity of culture. Putative pure cultures were Gram stained to ensure presence of Gram negative curved rods. Once pure cultures were established, cells were frozen at –80°C for long term storage (see section 2.2.6).

2.2.12 Bio-accumulation by freshwater mussels

The ability of the freshwater mussel (*Hyridella menziesi depauperta*) (Figure 2.2) to accumulate environmental *Campylobacter* species from the Avon and Heathcote Rivers was evaluated.

2.2.12.1 Mussel collection

Freshwater mussels were collected from Lake Coleridge, a high country inland lake in the central South Island. Mussels were transported to the laboratory in a container of lake water. Water samples were taken from the lake to ensure the absence of *Campylobacter* from both mussels and surrounding water. Mussels were stored in the Department of Zoology aquarium in freshwater circulating tanks maintained at 10°C until required.

2.2.12.2 Feeding of mussels

Mussels were fed weekly on suspensions of algae. Algal cultures of *Chlamydomonas spp.* or *Chlorella vulgaris* were prepared in Bold's basal medium (BBM) as described in appendix I. Mussels were regularly supplemented with a milk-solids based food. Mussel food supplement consisted of 'Complan', a proprietary human dietary supplement, with ground alfalfa and yeast in dry weight ratios of 0.02 alfalfa: 0.03 brewers yeast: 0.95 Complan (Roper and Hickey, 1995).

2.2.12.3 Preparation of mussels

Mussels in freshwater storage tanks were initially tested to demonstrate that they were not originally infected with *Campylobacter*. Flesh and shellwater was tested for *Campylobacter* as described in section 2.2.10.4. Mussels had an average size of 6.08cm in length, and an average flesh weight (including shellwater) of 13.07g. For river placement, four mussels were placed into 25 × 25cm-1cm-mesh bags. Each bag was sealed at the opening by threading string through the mesh and tying thoroughly in order to prevent loss of animals. Ten mesh bags containing four mussels were prepared.

2.2.12.4 River placement

Five bags were placed in the Avon River site (see section 2.2.9.1.1) and five placed in the Heathcote River site (see section 2.2.9.1.2). Samples were placed along each river site in ten metre intervals at low tide, to ensure that mussels would be fully submerged in water at all times. Mesh bags were firmly tied to 1.5m wooden stakes driven into the sediments and positioned below the water surface but above the sediments. Bags were left undisturbed for 48h at which time they were removed from the river and transported to the laboratory for processing.

2.2.12.5 Processing of mussels

Mussels were processed individually by enrichment in 100ml of Exeter enrichment broth as described in section 2.2.10.4. Putative *Campylobacter* colonies on post-enrichment CCDA plates were confirmed by Gram stain and purified by streak isolation (see section 2.2.11). Pure cultures were stored at –80°C as described in section 2.2.6.

2.3 Results

2.3.1 Minimum detection limits of passive filtration

Results from the detection limits of the passive filtration technique are shown in Table 2.3. This method does not provide quantitative data. However, the *C. jejuni* cfu/ml quantified from culturable counts of the cell suspension used for water inoculation are shown for each duplicate water sample.

Table 2.3: Sensitivity of the passive filtration technique shown by culturable detection limits of *C. jejuni* F38011 from seeded water

Treatment	Concentration (cfu/ml) in seeded water sample	Recovery of *C. jejuni* Replicate 1	Recovery of *C. jejuni* Replicate 2
1	4×10^6 cfu/ml	$+^A$	$+^A$
2	4×10^4 cfu/ml	$+^A$	$+^A$
3	4×10^2 cfu/ml	$+^A$	–
4	4×10^0 cfu/ml	–	–
5	0	–	–

+ = *C. jejuni* recovered – = *C. jejuni* not recovered
^{A} = *C. jejuni* confirmed by Gram stain and positive hippurate hydrolysis test

These results indicate that passive filtration can detect as low as 400 cells per ml in a 250ml water sample at least 50% of the time. However, this method does not appear to detect *C. jejuni* at low concentrations that would be expected in environmental water. A 100% detection rate required concentrations of $\geq 1 \times 10^7$cells.

2.3.2 Minimum detection limits of the sample enrichment method

Results from the detection limits of the enrichment broth method are shown in Table 2.4. This method does not generate quantitative data and was tested solely for its ability to isolate environmental *Campylobacters*.

Table 2.4: Sensitivity of the sample enrichment technique as determined by culturable detection limits of *C. jejuni* KLC 4235 from post-incubation broths

Treatment	Initial cell concentration (cfu/100ml) in seeded broth	Recovery of *C. jejuni* Replicate 1	Recovery of *C. jejuni* Replicate 2	Recovery of *C. jejuni* Replicate 3
1	1.733×10^{5}	+	+	+
2	1.733×10^{4}	+	+	+
3	1.733×10^{3}	+	+	+
4	1.733×10^{2}	+	+	+
5	1.733×10^{1}	+	+	+
6	1.733×10^{0}	+	+	+
7	1.733×10^{-1}	–	–	–
8	1.733×10^{-2}	–	–	–
9	1.733×10^{-3}	–	–	–
10	1.733×10^{-4}	–	–	–
11	0	–	–	–

+ = *C. jejuni* recovered – = *C. jejuni* not recovered
[A]= *C. jejuni* confirmed by Gram stain and positive hippurate hydrolysis test

Incubation of seeded enrichment broths can detect as low as 1.733 cells introduced to broth from an initial sample. This is the case in 100% of samples (all three replicates). This method is highly sensitive for detection of *C. jejuni* when compared to passive filtration (Table 2.3 above). This is most evident at lower cell concentrations where enrichments show a superior detection rate. Therefore, enrichments were used for all subsequent experiments.

2.3.3 Bio-accumulation using freshwater mussels

Isolation of environmental *Campylobacters* from the Avon and Heathcote Rivers using the filter feeding strategy of the freshwater mussel was successful. A total of eight *Campylobacter* isolates were purified from post-incubation CCDA plates of the 40 mussels tested. Of these eight isolates, seven were purified from 20 mussels placed in the Avon River and one from 20 mussels placed in the Heathcote River. Mussels screened for the presence of *Campylobacter* prior to river placement were

free of culturable *Campylobacter spp* (data not shown). As 20 mussels were placed in each river, *Campylobacter* purification rates equate to 35% and 5% respectively for the Avon and Heathcote Rivers. These purification rates, however, are not a true indication of isolation rates or detection rates. CCDA plates cultured with freshwater mussel enrichments from the Heathcote River were overgrown with competitive microflora. This made visualisation of typical *Campylobacter* colonies and typical *Campylobacter* cell morphology from Gram stains extremely difficult. At least five *Campylobacter* isolates confirmed by Gram stain (four from the Heathcote River) were 'lost' through lack of growth on successive streak isolation plates. If these are taken into consideration, true isolation rates may be as high as 40% and 25% for the Avon and Heathcote Rivers respectively.

In order to assess whether post-enrichment CCDA plates contained culturable *Campylobacter* that could not be visualised due to an excess of competitive microflora, growth from each CCDA plate was used to prepare a whole cell lysate for use as DNA template in a polymerase chain reaction (PCR) of the flagellin A (*flaA*) gene. Whole cell lysates were prepared as described in section 3.2.7.1. *FlaA*-PCR was performed as described in section 3.2.2 using the primers pg50 and pg3. *Campylobacter* DNA was detectable in 16 out of 18 plates tested from mussel enrichments from the Heathcote River. This is a detection rate of 88.8%. Post-incubation CCDA plates from enrichments of mussels obtained from the Avon River had little or no growth of competitive microflora. A number of plates yielded no growth at all. Assessment of *Campylobacter* accumulation by freshwater mussels was not quantitative and quantities of *Campylobacter* in each river could not be assessed. However, isolation and detection results confirm the presence of culturable *Campylobacter* species in both the Avon and Heathcote Rivers.

Water samples were taken from each river at the time of removing mussels from the rivers. *Campylobacter* was not isolated from the Avon River water sample. Post-enrichment CCDA plates from the Heathcote River water sample were overgrown with competitive microflora similar to that of mussel enrichment plate cultures. Although *Campylobacter* could not be purified from these plates, *Campylobacter*

DNA was detectable by *flaA*-PCR from a whole cell lysate prepared from bacterial growth on CCDA plates.

2.3.4 Environmental sampling results

Environmental *Campylobacter* isolation rates were generally low. A total of 22 *Campylobacter* isolates were purified from environmental sampling (including those isolated through freshwater mussel bio-accumulation experiments above) (see table 2.5).

2.3.4.1 Freshwater

A total of 30 freshwater samples were tested for the presence of *Campylobacter spp.* Of these 30 samples, 12 were taken from the Avon River, 16 from the Heathcote River and 2 from the Groynes recreational area. Five out of the 12 (41.7%) samples taken from the Avon River site were culture positive for *Campylobacter.* Five out of the 16 (31.25%) samples taken from the Heathcote River site were culture positive for *Campylobacter.* Isolation of *Campylobacter* from these rivers was consistent over the sampling period (July 1999-April 2000) and not confined to a specific sampling date or locality. Two out of two (100%) water samples taken from the Groynes recreational area were culture positive for *Campylobacter.* Water sampling from this site was restricted to a single sampling date for both water samples. As the Groynes was not used as a major sampling site, consistency of isolation and persistence of *Campylobacter spp.* in the water cannot be inferred.

2.3.4.2 Estuarine water

No environmental *Campylobacter* strains were isolated from water samples taken from the Beechville Rd jetty site or the South Brighton estuary site. This was surprising given the high isolation rates from both the Avon and Heathcote Rivers, which empty into the estuary and the abundance of bird life within the estuary. A total of 23 water samples were taken from the two estuary sites (10 from the Beechville Rd site and 13 from the South Brighton estuary site).

2.3.4.3 Seawater

One out of ten (10%) of the seawater samples taken from the Sumner-Cave Rock site was culture positive for *Campylobacter.* This was from the water sample taken on 20 September 1999. This water sample was taken at low tide directly in front of a large Blue-Shelled mussel bedrock. This particular sampling date was on a warm early spring afternoon when many members of the public were using the water for recreational swimming.

2.3.4.4 Marine mussels

No environmental *Campylobacter* strains were isolated from Blue-shelled mussels taken from the Sumner-Cave Rock site. A total of 26 mussel samples were taken during the course of this study. Although the water sample taken from this site on the 20 September 1999 was positive for *Campylobacter*, four separate mussel samples taken at the same time were negative.

2.3.4.5 Cockles

One out of 30 (3.3%) cockle samples was positive for *Campylobacter.* This sample was taken from the Beechville Rd Jetty site at low tide within a seagull bathing area on 26 March 2000. Three other cockle samples taken on the same date were negative for *Campylobacter.* A water sample taken directly from the cockle sampling region was also negative for *Campylobacter.* This cockle sampling site is regularly visited by members of the public gathering cockles for consumption (Calder 1998).

2.3.4.6 Mudflat snails

No *Campylobacter* strains were isolated from any snails sampled from the South Brighton estuary site. Throughout the snail sampling period, 12 samples were taken in total. The lack of positive results led to another set of experiments detailed in section 3.2.9.

Collectively, these results show a highly variable incidence of *Campylobacter* species from environmental samples. Freshwater was found to yield significantly higher isolation rates than estuarine or seawater. The combined *Campylobacter* isolation rate from freshwater samples was 40% (12 out of 30). This compares to a combined salt-

water isolation rate of just 3% (1 out of 33). All water tested was of a relatively low turbidity and could be processed with ease using membrane filtration. Water from the Heathcote River was of the highest turbidity from all water types sampled. Isolation of *Campylobacter spp.* from cockles and mussels did not correlate well with the surrounding water. On the sole occasion that *Campylobacter* was isolated from cockles, the surrounding water was negative for isolation. Conversely, the one occasion that *Campylobacter* was isolated from seawater, the mussels submerged in the water were negative for isolation. Environmental sampling experiments were purely qualitative and not intended to quantify *Campylobacter* cell numbers in environmental samples.

2.3.5 Environmental *Campylobacter* isolate descriptions

Table 2.5 lists the 22 *Campylobacter* isolates purified from environmental samples and indicates sampling locality and dates.

Table 2.5: *Campylobacter* isolates recovered from the environment through sampling

Isolate code[A]	Sample type	Location	Isolation date
AW9901	Freshwater	Botanic Gardens Bridge; Avon River	24 August 1999
HW9902	Freshwater	Aynsley Terrace; Heathcote River	20 September 1999
SW9903	Seawater	Sumner-Cave rock; Pacific Ocean	20 September 1999
AW9904	Freshwater	Hospital Bridge; Avon River	10 December 1999
AW9905	Freshwater	Botanic Gardens Bridge; Avon River	10 December 1999
HW0006	Freshwater	Aynsley Terrace; Heathcote River	15 February 2000
AW0007	Freshwater	Mid-Site; Avon River	15 February 2000
AW0008	Freshwater	Mid-site; Avon River	15 February 2000

Table 2.5: Continued...

Isolate code[A]	Sample type	Location	Isolation date
EC0009	Cockle	Beechville Road Jetty; Estuary	26 March 2000
HW0010	Freshwater	Aynsley Terrace; Heathcote River	3 April 2000
HW0011	Freshwater	Aynsley Terrace; Heathcote River	3 April 2000
HW0012	Freshwater	Aynsley Terrace; Heathcote River	3 April 2000
GW0013	Freshwater	Swing Bridge; The Groynes	3 April 2000
GW0014	Freshwater	Swing Bridge; The Groynes	3 April 2000
AM0015	Freshwater Mussel	Mussel No.1 (bag 1); Avon River	11 May 2000
AM0016	Freshwater Mussel	Mussel No.10 (bag 3); Avon River	11 May 2000
AM0017	Freshwater Mussel	Mussel No.12 (bag 3); Avon River	11 May 2000
AM0018	Freshwater Mussel	Mussel No.13 (bag 4); Avon River	11 May 2000
AM0019	Freshwater Mussel	Mussel No.14 (bag 4); Avon River	11 May 2000
AM0020	Freshwater Mussel	Mussel No.17 (bag 5); Avon River	11 May 2000
AM0021	Freshwater Mussel	Mussel No.19 (bag 5); Avon River	11 May 2000
HM0022	Freshwater Mussel	Mussel No.14 (bag 4); Heathcote River	11 May 2000

A: The first letter of isolate codes refers to the sampling site; A = Avon River, H = Heathcote River, S = Sumner-Cave Rock site, E = Estuary, G = The Groynes recreational area. The second letter of isolate codes refers to sample type; W = Water, C = Cockle, M = Mussel. The first two digits of isolate codes refer to year of isolation; 99 = 1999, 00 = 2000. And the final two digits of isolate codes represent the isolate number.

2.4 Discussion

2.4.1 Recovery of *C. jejuni* from seeded water samples

Comparison of passive filtration with the selective enrichment method demonstrated enrichments to have a superior sensitivity for recovery of *C. jejuni*. With passive filtration, an absolute minimum of 1×10^5 cells in a water sample was required for detection. As filtration of water samples is expected to collect most if not all bacterial cells onto the surface of the membrane, 1×10^5 cells would need to be present in a water sample regardless of the volume of the sample. Recreational surface waters are assumed to contain low cell numbers in many cases (Hudson et al., 1999). Hence, for passive filtration to be effective, particularly large volumes of environmental water would be required to obtain the absolute minimum numbers of cells for detection of *Campylobacter*. Processing of such large volumes of water is usually not a viable option as membrane filters would become severely clogged with particulate matter and non-target microflora. This would make movement of *Campylobacter* cells through the pores of the membrane increasingly difficult. Passive filtration has been found to be useful for isolation of *Campylobacter* from clinical specimens (Steele and McDermott, 1984; Kiehlbauch et al., 1996; Lopez et al., 1998). In these studies, faecal suspensions prepared from stool samples from patients presenting with acute diarrhoea were used for passive filtration. Stool samples from patients with diarrhoea as a result of *Campylobacter* infection would be expected to contain high numbers of *Campylobacter* cells. Therefore, passive filtration would be sufficiently sensitive for isolation (but not quantitation) of clinical *Campylobacter spp.* However, passive filtration has also been successfully used for isolation of *Campylobacter* from environmental water samples (Arvanitidou et al., 1995; Carter et al., 1987). Arvanitidou et al. (1995) were able to culture *Campylobacter* from 16.3% of water samples analysed from river and lake water. Blaser and Cody (1986) used a modification to the passive filtration method by placing membranes face down on selective agar. This process was found to be more sensitive than the use of Exeter or Preston enrichment broth for *Campylobacter* recovery from seeded water. For environmental samples however, this method would substantially increase the recovery of competitive microflora, which may overgrow *Campylobacter* species.

The primary question for the detection limits of enrichment broth method was 'what is the minimum number of cells required to be introduced to enrichment broths from any environmental sample for isolation of *Campylobacter* species?' Exeter enrichment broth was able to detect a minimum of 1.7 cfu per seeded sample (Table 2.4). Post-filtration membranes from seeded water were not used for the enrichment method. This was due to the fact that aquatic invertebrates could also be processed using this method. The result suggests that approximately two cells per sample are all that is required for isolation after enrichment. This value is probably not a true indication of the detection limits of this method from environmental samples. This is because the experiment was conducted using sterile broth seeded with a pure culture of *C. jejuni*. In environmental samples, the presence of non-target microflora may out-compete the growth of *Campylobacter spp.* and result in a reduction of numbers. Similarly, inhibitory effects of the sample itself (eg., invertebrate flesh, see section 3.3.9) could reduce or prevent the growth of *Campylobacter* in enrichment broths. Furthermore, cell suspensions were made in PBS. Therefore, considerably more than the minimum 1.7 cells would be required for *Campylobacter* isolation from environmental samples. Previous studies addressing the detection limits of enrichment broth methods have given variable results. Humphrey (1986) reported a detection limit of one *C. jejuni* cell from raw milk samples when a pre-incubation period was used. In contrast, Armstrong (1997) found a minimum of 20 cells were required for Exeter broth detection from seeded water. This value is more than 10 times higher than the detection limit for this study. Although passive filtration may be useful for obtaining *Campylobacter* from clinical specimens, the results of this study clearly indicate that use of Exeter enrichment broth is significantly more sensitive for recovery of *Campylobacter*. It was for this reason that enrichment of samples in Exeter broth was the preferred method for all subsequent experiments.

2.4.2 Water sampling

Isolation of *Campylobacter spp.* from water was generally low with 13 out of 63 (20.6%) of water samples positive for *Campylobacter*. Isolation rates were highly variable and dependent on the water type sampled. Freshwater samples produced the highest *Campylobacter* isolation rates with a combined total of 12 positive samples out of 30 (40%) tested from the Avon and Heathcote rivers and the Groynes

recreational area. The large bird populations at each of these sampling sites are likely to be the major source of *Campylobacter* dissemination into the water. Surprisingly, Calder (1998) was unable to recover *Campylobacter spp.* from either the Avon or the Heathcote Rivers. It was suggested that numbers of *Campylobacter spp.* present were below the detection limits of the methodology used. Given that the methodology used for that study was identical to the methodology used in the current study, it may be possible that the bacterial dynamics of both rivers have changed between 1997-1998 and 1999-2000. This may be due to an increase in the incidence of *Campylobacter* in gulls and waterfowl resulting in increased *Campylobacter* contamination of the rivers. Alternatively, run-off from stormwater drains entering the rivers may have become increasingly contaminated with *Campylobacter* and hence contributed to increased *Campylobacter* in each river. Sampling for both studies was done over a similar time period eliminating any variability in isolation due to seasonal fluctuations in detection rates. Recent studies by the Institute of Environmental Science and Research (ESR) have also reported much higher isolation rates from local rivers than reported by Calder (1998), (J. Klena, personal communication).

Campylobacter isolation rate for water samples from the Groynes recreational area was 100%. However, the number of samples taken was small (two samples) and therefore does not give a clear indication of *Campylobacter* contamination of this site. Armstrong (1997) used a combination of enrichment and passive filtration for isolation of *Campylobacter* from the Groynes. In this study, seven out of 26 (29.6%) of water samples were positive for *Campylobacter* using either method (isolation rates from passive filtration and enrichments were 12.5% and 50% respectively). These values are likely to be more indicative of *Campylobacter* isolation rates from the Groynes, had this area been used as a major sampling site. The results of the current study show that freshwater from the Avon and Heathcote Rivers and possibly the Groynes recreational area are a potentially important source for *Campylobacter* species in the environment. Other studies addressing freshwater reservoirs of *Campylobacter* have given relatively variable results. Bolton et al. (1987) found *Campylobacter spp.* in 43% of river water samples. Pianetti et al. (1998) reported 30.9% of all samples taken from rivers in Italy were positive for *Campylobacter* while Brennhovd et al. (1992) found 43.8% of water samples from Norway yielded

Campylobacter. Martikainen et al. (1990) addressed the seasonal occurrence of *Campylobacter* in urban surface waters and found isolation rates of 5-6% in summer and winter with 17-24% in spring and autumn. A particularly high isolation rate was reported from the canals of Bangkok with 116 out of 156 (74.3%) of water samples positive for *Campylobacter* (Dhamabutra et al., 1992). The methodology used, seasonal fluctuations or prevailing environmental conditions could account for some of this variability. Despite the differences in isolation rates reported from freshwater, most studies appear to show a freshwater *Campylobacter* isolation rate of between 5-45%. The current study correlates well with previous isolation reports.

Isolation of *Campylobacter spp.* from estuarine and seawater was considerably lower than freshwater with only one out of 33 (3%) of samples giving a positive result. Post-enrichment CCDA plates from freshwater samples tended to be overgrown with competitive microflora making purification of *Campylobacter* colonies difficult. Estuarine and seawater samples, however, often showed little or no growth of contaminating microflora. Two major possibilities could account for this lack of bacterial growth. Firstly, the selective antibiotics used in Exeter enrichment broth may have been sufficient for elimination of most if not all competing microflora. Incidentally, the use of such antibiotic cocktails has been thought to also inhibit the growth of *Campylobacter* species aside from *C. jejuni* (eg., *C. lari*, *C. upsaliensis* and to a lesser extent *C. coli*) (Ng et al., 1988; Steele and McDermott, 1984). Although this may be likely, not all post-enrichment plates were free of competing microflora, with a few plates showing prolific microbial growth. The second possibility is that the flow rate of seawater and substantial horizontal and vertical mixing of water coupled with the regular flow of water in and out of the estuary with the tides would rapidly remove bacterial cells deposited in the water (Calder, 1998). This is more likely to be the case as all water sampling was done at mid or low tide when the majority of the water along with its accompanying bacterial loads would be washed out to sea. Furthermore, bacterial numbers present in seawater were found to decrease at a faster rate than those in freshwater when exposed to natural sunlight (Davies and Evison, 1991). *Campylobacter* cells in estuarine and seawater may be more susceptible to UV light damage. Isolation rates of *Campylobacter* species from seawater from three beaches in Tel-Aviv were found to range from 5-38% (Ghinsberg

et al., 1994). Obiri-Danso et al. (1999) reported isolation rates of between 20-100% from beaches in England while Alonso and Alonso (1993) found 13% of marine water samples from Spain yielded *Campylobacter*. The prevalence of *Campylobacter spp.* in marine waters appears to be much more variable than that of freshwater. A number of factors are potentially responsible for these dramatic variations in *Campylobacter* isolation rates. These include the sampling location, the isolation methodology and the nature of the prevailing environmental conditions samples were obtained from (Calder, 1998).

2.4.3 Aquatic invertebrate sampling

Isolation of *Campylobacter spp.* from aquatic invertebrates present in and around the Avon-Heathcote Estuary was relatively unsuccessful. Only one out of 68 (1.47%) of a combination of invertebrate samples (cockles, mussels, snails) were positive for *Campylobacter spp.* Primary post-enrichment CCDA plates were overgrown with competitive microflora making identification of typical *Campylobacter* colonies difficult. Four cockles or mussels were processed as one sample. It was suspected that the use of this level of flesh mass was introducing an excess of non-target bacteria to Exeter enrichment broths resulting in the overgrowth of these organisms on post-enrichment CCDA plates. To test this, the cockles and mussels collected on the final sampling date of this study were processed individually in an identical volume (100ml) of enrichment broth. Although this did not appear to decreases the prolific growth of competitive microflora on some CCDA plates, the one *Campylobacter spp.* isolated was when shellfish were processed individually. Therefore, it is recommended that a variety of invertebrate flesh weights or diluent (broth) volumes be used when such invertebrate sampling is undertaken.

The *Campylobacter spp.* isolated from invertebrate sampling was from a cockle taken at the Beechville Rd Jetty site during low tide. The pools of water that exist at low tide are frequently used as bathing water by many birds. At this time, dissemination of avian *Campylobacter spp.* into the water is likely. However, *Campylobacter spp.* are unlikely to be accumulated by underlying cockles who only feed during high tide (Stephenson, 1981). The incoming high tide would dilute and wash away much of the *Campylobacter* point sources introduced by birds. However, it is not inconceivable

that some cockles may accumulate and concentrate *Campylobacter* cells that have settled on the surface of the sediments and are resuspended in the water by the incoming tide. For this to be the case, the *Campylobacter* cells accumulated at high tide would have to be retained within the cockle until low tide when sampling was done. It is not known whether *Campylobacter spp.* are capable of infecting and colonising cockle flesh. It is assumed that bacterial cells are normally used as a food source and as such, are eliminated from digestive tissues relatively quickly. Abeyta et al. (1993) discussed that *Campylobacter* species have routinely been found in cockles from the Washington coast, however isolation rates and sampling conditions were not given.

Campylobacter spp. were not isolated from Blue-shelled mussels. This was also found to be the case for Blue-shelled mussels tested by Calder (1998). In contrast, to cockles, mussels tend to feed throughout the day and night and therefore could continuously accumulate bacterial pathogens from point sources as they arise. Mussels collected from the Sumner-Cave Rock site are almost completely uncovered at low tide and were seldom observed to be feeding. Care was taken to sample only those mussels submerged in the water at low tide. It was expected that these mussels would have been feeding immediately prior to sampling, although this could not always be confirmed due to difficulties with clear visualisation of mussels under the water. Studies addressing the presence of *Campylobacter spp.* in mussels have produced highly variable results. Wilson and Moore (1996) reported 42% of shellfish positive for *Campylobacter* at harvest, while Ripabelli et al. (1999) were unable to isolate *Campylobacter* from mussels in Italy. The transient existence of *Campylobacter* in the marine environment suggests that its incidence in mussels is dependant on the frequency and size of point sources in shellfish-gathering waters (Abeyta et al., 1993).

Snails were found to contain many bacteria as determined by the considerable growth and variation in colony types on post-enrichment CCDA plates. However, no *Campylobacter* species were isolated. The presence of *Campylobacter spp.* in snails does not appear to have been well studied. Luechtefeld and Wang (1981) sampled snails collected from a freshwater stream for *C. jejuni*, but were unable to isolate any

Campylobacter from 50 specimens. Estuarine snails tend to be grazers of algae and other particulate matter present on the surface of sediments and may accumulate *Campylobacter spp.* that settle onto intertidal sediments. The *Campylobacter* accumulation potential of these snails is relatively unknown. The accumulation efficiency and survival of *C. jejuni* in estuarine snails is addressed in Chapter III.

The presence of *Campylobacter spp.* in water did not correlate well with their prevalence in the aquatic invertebrates tested. When *Campylobacter* was isolated from seawater, four out of four mussel samples taken from the same area were negative. It is assumed that this *Campylobacter* isolate was from a point source collected in the water sample before it could reach the mussels. Subsequent seawater samples were negative for *Campylobacter*, suggesting the transient nature of this source of *Campylobacter*. When *Campylobacter* was isolated from a cockle sample, the surrounding water was found to be negative. This was unexpected given the use of this water by birds for bathing. Although the bacterial contaminants present in aquatic invertebrates are thought to closely indicate the nature of the surrounding environment (due to indiscriminate feeding patterns), *Campylobacter spp.* appear to be so highly variable in their presence or absence within marine waters that correlations between existence in water and naturally occurring fauna is inconsistent.

2.4.4 Bio-accumulation by freshwater mussels

Natural filter feeders such as mussels have successfully been used to assess water quality (Donnison and Ross, 1999). Natural bio-accumulators are preferred for indirect monitoring of water because they are thought to concentrate bacterial pathogens and retain them for certain periods of time (Plusquellec et al., 1994). This gives information not only on the instantaneous contamination of the surrounding water, but also to a certain degree the immediate history of the contamination of water (Plusquellec, et al., 1994). The current study found freshwater mussels to be efficient accumulators of *Campylobacter spp.* from both the Avon and Heathcote rivers. Although more *Campylobacter* isolates were purified from mussels placed in the Avon River than the Heathcote River, this was not an indication of the level of *Campylobacter* contamination in each river. In fact, the Heathcote River was found to contain a higher level of bacterial contamination than the Avon River. This was

demonstrated by the high numbers of competitive microflora on post-enrichment CCDA plates making purification of *Campylobacter spp.* difficult.

An important distinction was made between *Campylobacter* purification, isolation and detection rates. The purification rate was used to refer to the number of isolates that were obtained in pure culture after successive streak isolations. These isolates are desirable because they can subsequently be used for speciation and characterisation. A total of seven out of 20 *Campylobacter spp.* from the Avon River and one out of 20 from the Heathcote River were purified from mussel enrichments. The isolation rate was used to refer to the number of mussel enrichments yielding putative *Campylobacter* colonies on post-enrichment CCDA plates confirmed as such by Gram stain. The isolation rate includes the number of purified isolates along with those isolates that were 'lost' during successive streak isolations. Purification of *Campylobacter spp.* from post-enrichment CCDA plates from Heathcote River mussels proved difficult. This was primarily due to the overgrowth of competitive microflora. *Campylobacter spp.* normally take 24-48h to form visible colonies on CCDA plates, whereas the competitive microflora grew prolifically often in less than 12h. This prolific growth of contaminating bacteria continually overgrew *Campylobacter* colonies and resulted in loss of the isolate through successive streak isolations. PCR was used to detect *Campylobacter*-specific DNA from highly contaminated CCDA plates. The number of plates that yielded PCR detectable *Campylobacter* DNA resulted in the detection rate. Four isolates from the Heathcote river and one from the Avon River were 'lost' through successive streak isolations. If these are taken into account, isolation rates of 40% for the Avon River and 25% for the Heathcote River are obtained. These are comparable to the isolation rates of 41.7% and 31.25% obtained from water sampling of the Avon and Heathcote Rivers respectively. The PCR detection rate for *Campylobacter* present in the Heathcote river was 88.8%. If the one isolate purified from a Heathcote river mussel is included, a detection rate of 89.5% arises. This suggests that the Heathcote River is highly contaminated with *Campylobacter spp.* along Aynesly Tce. Although freshwater mussels confirm the presence of *Campylobacter* in the Avon and Heathcote Rivers, the result is only qualitative and numbers of cells present in the rivers cannot be enumerated. Given that mussels are thought to clear bacterial cells from their

digestive tract relatively quickly, it is conceivable that the water would have only recently been contaminated by *Campylobacter*.

Donnison and Ross (1999) have also been successful in recovery of *Campylobacter* from freshwater mussels placed in rivers. *Campylobacter* was recovered from four out of five water sites with an isolation rate of nine out of 17 (52.9%) of mussel samples positive for *Campylobacter spp.* The efficient accumulation of *Campylobacter spp.* by freshwater mussels placed in rivers makes them an ideal candidate as a bio-indicator. This is particularly true for freshwater where a lack of naturally occurring filter feeders is evident. In later sections of this thesis, the usefulness of the freshwater mussel as a bio-indicator is further tested by comparison of *C. jejuni* detection rates in water with detection rates in mussel flesh.

Chapter III

Invertebrate Uptake and Detectability of *C. jejuni*

3.1 Introduction

The difficulties involved with detecting *Campylobacter spp.* from water sources implicated in disease outbreaks and sporadic cases make the use of an invertebrate biological indicator a potentially important method of *Campylobacter* detection. This is particularly true if the chosen bio-indicator can accumulate *Campylobacter* cells from a non-detectable concentration in water to a readily detectable concentration in the animal. A close correlation between survival of *Campylobacter* in water and the bio-indicator is of utmost importance when testing a potential bio-indicator.

3.1.1 Survival of *Campylobacter spp.* in water

The persistence of *Campylobacter spp.* in environmental water supplies is dependent on a number of variables. These include the water type, the species of *Campylobacter,* water temperature, nutrient depletion, competition from other organisms, oxygen stresses and UV-light (Thomas and Mabey, 1996; Korhonen and Martikainen, 1991; Davies and Evison, 1991; Terzieva and McFeters, 1991; Thomas et al., 1999; Chynoweth et al., 1998; Buswell et al., 1998; Harvey et al., 1996; Holler et al., 1998; Hazeleger et al., 1998; Mason et al., 1996; Mason et al., 1999; Knill et al., 1981). *Campylobacter* species tend to survive for longer time periods in freshwater than in seawater (The National Advisory Committee on Microbiological Criteria for Foods, 1995). This is largely due to their sensitivity to high concentrations of NaCl. A 1% concentration of NaC1 was found to significantly increase cell death rate while a 2% NaC1 concentration was bactericidal (The National Advisory Committee on

Microbiological Criteria for Foods, 1995). Different species of *Campylobacter* as well as different strains of the same species have been shown to survive in water for variable amounts of time. Thomas et al. (1999) addressed the survival of four species of thermotolerant *Campylobacter spp.* in water microcosms. *C. jejuni and C. lari* were reported to be the most resilient to environmental water stresses. *C. coli* and *C. upsaliensis* showed similar survival characteristics to each other but were less resilient than *C. jejuni* or *C. lari.* Survival of *Campylobacter spp.* is most dependent on the water temperature. Several studies have addressed the effect of temperature on survival of *Campylobacter* in water with similar results. Temperatures of between 4-6°C tended to prolong the survival of *Campylobacter* species when compared to higher temperatures (Holler et al., 1998; Chynoweth et al., 1998, Terzieva and McFeters, 1991). *Campylobacter* isolates held at 4°C and 10°C yielded mean survival times of 202 and 176h respectively whereas isolates incubated at 22°C and 37°C only showed mean survival times of 43 and 22h respectively (Buswell et al., 1998). Calder (1998) reported similar results where survival times as high as 984h at 4°C and 72h at 30°C were observed. At 5°C, *Campylobacter spp.* have been shown to survive for up to 28 days (Thomas and Mabey, 1996). As temperatures of 5-15°C are found in environmental waters in temperate regions, the persistence of *Campylobacter* in water of these temperatures could be important for transmission to the human population.

Although temperature is likely to be the most important factor dictating *Campylobacter* survival in water, other variables may simultaneously have an effect. One such variable is nutrient availability. *Campylobacter* cells placed in water microcosms containing natural river water show a significantly decreased rate of decay relative to those placed in de-ionized water (Thomas et al., 1999). The lack of nutrients present in water is important for the maintenance of *Campylobacter* cells. Competition between other microorganisms in water is another potentially important variable for *Campylobacter* survival. Korhonen and Martikainen (1991) reported that *C. jejuni* survived better in filtered lake water than in untreated water, highlighting the inhibitory effects of competitive microflora. The preference of *Campylobacter spp.* for microaerophilic conditions makes them particularly susceptible to oxygen stresses in environmental waters. Oxygen stress has been shown to result in poor survival of *Campylobacter spp.* (Harvey et al., 1996). The methods generally used to measure

Campylobacter survival in water are culturable counts or direct viable counts (DVC). However, the loss of viability of bacteria (as judged by membrane changes) using direct viable counts is usually slower than the loss of culturable bacteria (Garcia-Lara et al., 1991). This suggests that *Campylobacter*, like many other non-aquatic bacteria in water may lose culturability, but remain viable.

3.1.2 The viable but non-culturable (VNC) state of *Campylobacter spp.*

The possibility of *Campylobacter spp.* to exist in the environment in a viable state but become unculturable has profound implications for public health if these cells retain their ability to become virulent. In effect, environmental sources implicated in outbreaks and sporadic cases of campylobacteriosis would escape detection of *Campylobacter spp.* by traditional culture methods. The high number of variables simultaneously acting upon *Campylobacter spp.* shed into environmental waters may cause sufficient stress to induce cells into entering a viable but non-culturable (VNC) state. VNC cells have been defined as 'bacteria that are metabolically active, while being incapable of undergoing the cellular division required for growth (Koenraad et al., 1997). It has been suggested that the frequent exposure of *Campylobacter* cells to stresses such as temperature, starvation, osmotic stress, oxygen tension and UV-light in the environment are responsible for onset of the VNC state (Davies and Evison, 1991; Garcia-Lara et al., 1991; Harvey et al., 1996; Reezal et al., 1998). A number of human pathogens have been identified as possessing the ability to enter a VNC state. These include *Vibrio cholerae, C. jejuni, E. coli, Helicobacter pylori* and *Salmonella enteritidis* (Rollins and Colwell, 1986; Colwell et al., 1996; Ozkanca, 1996; Mitchell, 1999, Barer, 1997; Tholozan et al. 1999).

The onset of a VNC state has been suggested to be a survival mechanism in response to harsh conditions, similar to that of spore formation (Barer, 1997). Spore forming bacteria are capable of reverting to the vegetative cell growth phase when favourable conditions are encountered. For VNC bacteria to be analogous to sporulating bacteria, they must also revert to a culturable state when favourable conditions are encountered. Whether VNC cells are capable of such 'resuscitation' or simply represent an early stage of cell death has been the topic of much debate (Barer, 1997; McDougald et al., 1998). With respect to bacterial pathogens such as *Campylobacter*

spp., cells in the VNC state must retain their ability to cause disease if they are to maintain significance as a public health threat. Researchers have gathered some evidence that supports the concept of virulent VNC bacteria and their resuscitation to culturable cells. Jones et al., (1991) showed that *C. jejuni* cells that became non-culturable in pond water remained capable of colonisation if fed to suckling mice (as shown by enteric disease onset). Similarly, Saha et al., (1991) demonstrated that freeze-thaw induced VNC cells could be cultured after passage through a rat gut. In a controlled human volunteer study, VNC cells of *V. cholera* were also found to regain culturability and pathogenesis (Colwell et al., 1996). Alternatively, Beumer et al., (1992) were unable to resuscitate *C. jejuni* administered orally to laboratory rabbits, mice or human volunteers. Boucher et al., (1994) suggested that due to the fact that VNC cells of *C. jejuni* were unable to maintain metabolic activity in adverse conditions, they represent a degenerative stage rather than a true dormant (VNC) state. The identification and characterisation of possible genetic determinants that regulate onset of the VNC state will give definitive evidence of a true programmed response (McDougald et al., 1998). If *Campylobacter spp.* entering aquatic environments do enter a VNC state and passage through an animal induces resuscitation, the use of invertebrate bio-indicators would become a highly desirable method for detection.

3.1.3 Correlation of *Campylobacter spp.* with indicator bacteria

Due to the difficulties associated with the isolation and identification of *Campylobacter spp.* from environmental water samples, the use of an appropriate indicator organism would be a preferred method of detection. However, standard faecal indicator bacteria do not sufficiently represent the presence of *Campylobacter spp.*

Early reports of *Campylobacter spp.* isolation from water suggested a close correlation with presence of *E. coli.* Knill et al., (1978) found that thermophilic *Campylobacter spp.* were isolated only in the presence of *E. coli.* A subsequent study by Bolton et al. (1982) confirmed this initial finding. However, no correlation between numbers of *Campylobacter spp.* and *E. coli* was evident. Alonso and Alonso's (1993) study strengthened these reports where thermotolerant

Campylobacter spp. were routinely isolated in the presence of faecal coliforms, although the levels of each organism did not correlate with one another. Carter et al., (1987) found considerable variation in faecal coliform densities in water samples from which *Campylobacter spp.* was isolated. Faecal coliform levels of between 0-104cfu/100ml were obtained, suggesting no obvious relationship between *Campylobacter* densities and indicator bacteria densities. The use of geometric means to correlate indicator bacteria with *Campylobacter spp.* also showed no significant difference between *Campylobacter* positive and negative water samples (Arvanitidou et al., 1995). In contrast, Brennhovd et al., (1992) reported a highly significant relationship between *Campylobacter* prevalence and faecal coliforms. The high level of variation in correlation of indicator bacteria with *Campylobacter spp.* presence in water obtained from each of the studies mentioned above suggest that faecal indicator bacteria do not predict the presence or absence of *Campylobacter spp.* in environmental waters.

The correlation of faecal coliform bacteria with *Campylobacter spp.* in shellfish is equally dubious. Wilson and Moore (1996) reported that *Campylobacter spp.* were isolated from 14% of shellfish that were free of faecal coliforms. Additionally, 21% of shellfish harbouring *Campylobacter* did not harbour any detectable *E. coli.* Therefore, the lack of predictability demonstrated by faecal coliforms, particularly regarding *Campylobacter spp.* presence, makes their use limited (Gale, 1996).

3.1.4 Accumulation of pathogenic bacteria by shellfish

It has been estimated that shellfish present in microbially contaminated water can concentrate bacterial numbers by 6.5-8.5 times depending on the bacterial count in the water (Timoney and Abston, 1984). This level of bacterial concentration arises through the normal feeding and water pumping processes of bivalve molluscs. The ability of shellfish to accumulate bacterial cells suspended in the water column is dependent on the shellfish species. Each shellfish species will possess its own unique physiological parameters for pumping rate and filtration efficiency (Calder, 1998). The amount of time in which bivalves spend feeding will affect the rate of bacterial accumulation. It has been estimated that at optimal temperatures in the natural environment, shellfish will spend as much as 95% of their time in feeding activity

(Boyle, 1981). The ventilation rate of shellfish refers to the flow of water through the animal. For an average 5cm mussel, the ventilation rate is considered to be approximately 1.5 litres per hour (Boyle, 1981). Timoney and Abston (1984) estimated that the ventilation rate for hard clams at 20°C is as high as 100ml per minute (6L/h).

Given the potentially high flow rate of water through shellfish, it is possible that *Campylobacter* cells present in environmental water at undetectable levels can be concentrated to detectable levels in shellfish tissue. Suspended particles as small as 1μm in diameter have been reported to be retained by mussels. A 100% retention rate has been achieved for suspensions of particles larger than 2μm in undisturbed mussels (Boyle, 1981). Environmental factors have been shown to significantly affect the retention or elimination of pathogenic bacteria (Rowse and Fleet, 1984).

Different bacterial species have been shown to possess variable shellfish accumulation and retention properties. Timoney and Abston (1984) observed *Salmonella enterica* serovar Typhimurium numbers declined less rapidly than *E. coli* at 24h post-inoculation of aquaria containing clams. Similarly, Son and Fleet (1980) observed *Bacillus cereus* counts remained stable in stored oysters while *Vibrio parahaemolyticus* counts increased prior to decreasing during the first four days of storage. Shellfish have a number of mechanisms in which to rid themselves of pathogenic bacteria during feeding. One such mechanism is the production of pseudofaeces. Particles that are not suitable for digestion due to composition or size are removed in pseudofaeces. Timoney and Abston (1984) reported high numbers of *Salmonella* and *E. coli* present in pseudofaeces. Bacterial counts as high as 2.6 x 10^6cfu of *E. coli* and 4.3 x 10^4cfu of *Salmonella typhimurium* were observed in pseudofaeces collected 6-24h after exposure to these pathogens. These bacteria were observed to be physically entrapped in the long thin mucosal strands of pseudofaeces. The physical binding of bacteria within pseudofaecal strands was found to be very stable over a pH range of 4.0 to 8.1 with no release of bacteria into the surrounding water. This suggests that faeces and pseudofaeces are effective for release of digested and undigested bacterial matter from the animal but do not contribute to the re-inoculation of overlying waters.

A number of shellfish also possess defence mechanisms such as production of haemocytes and tissue phagocytes to limit adherence and possible invasion of tissues by pathogenic bacteria (Friedl et al., 1992). However, bacterial particles that are capable of avoiding destruction by haemocytes and tissue phagocytes can persist for extended periods in shellfish (Friedl et al., 1992). In addition, *Vibrio spp.* have been observed to inhibit the filtration abilities of mussels (Birbeck et al., 1987). With respect to *Campylobacter* species, accumulation by shellfish has been found to be rapid. Arumagaswamy et al., (1988) found that oysters allowed to feed in water containing approximately 10^4 cfu/ml of *C. jejuni* or *C. coli* contained between 10^2 and 10^3 cells per gram of flesh within 1h of active feeding. However, no *Campylobacter* cells were detectable after 48h of depuration (placement in sterile water). Assuming *Campylobacter spp.* accumulation and elimination is similar in the freshwater mussel, this system could be a useful biological indicator for the presence of *Campylobacter spp.* in recreational water supplies.

3.1.5 Properties of an ideal invertebrate bio-indicator

For an aquatic invertebrate to be a good biological indicator allowing routine use for *Campylobacter spp.* detection, it must meet a number of specific criteria:

1. The isolation and identification of specific pathogens has traditionally been undesirable because these pathogens tend to enter water supplies sporadically and do not survive for a sufficient amount of time to facilitate detection. In effect, the presence of pathogens are under-represented in laboratory samples. Therefore to allow specific pathogen detection, a potential bio-indicator must have the ability to concentrate pathogenic cells to levels that are detectable by routine procedures.

2. The presence of pathogens in water supplies is often thought to be masked by cells entering a viable but non-culturable state. As VNC cells can not be grown on standard culture media, they escape detection. A candidate bio-indicator is therefore required to possess the ability to maintain cells in a culturable form for longer periods than the water.

3. Indicator organisms are usually chosen because their presence is evident in contaminated water whilst their absence is associated with unpolluted water. With respects to an invertebrate bio-indicator, the accumulation of the bacterial pathogen must be by a transient pattern. When the pathogen is present in the water, it is also present in the invertebrate. Conversely, when the water is free of the pathogen, the invertebrate bio-indicator is also free of the pathogen.

4. Traditionally, the quantity of an indicator organism tends to correlate with the amount of pollution (level of contamination). In an invertebrate bio-indicator, the persistence of the pathogen must correlate with its persistence in water. High bacterial loads in the water must correlate with higher bacterial loads in the bio-indicator or a higher number of positive samples.

5. The invertebrate bio-indicator must be capable of efficiently eliminating the pathogen from its tissues. Cells must not be capable of adhering to and invading the invertebrate flesh resulting in colonisation and infection.

If a candidate invertebrate bio-indicator can meet each of these criteria, it would be of considerable use for elucidating water sources of *Campylobacter spp.* as well as for providing valuable information as to the transmission source of disease outbreaks and potentially, sporadic cases. Moreover, it may give insights into the true level of *Campylobacter* contamination of recreational water if cells are maintained in a culturable state.

3.1.6 Objectives for invertebrate uptake and detectability of *C. jejuni*

Invertebrate uptake experiments were undertaken to determine the survival and detectability of *C. jejuni* within an *in vitro* (tank) water system for assessment of potentially suitable bio-indicators. The main objectives of these experiments were to:

1. Evaluate the most sensitive and appropriate method for obtaining nucleic acid template of a sufficient purity and yield for PCR detection of *Campylobacter*

2. Assess survival and detectability of *C. jejuni* from seeded freshwater over a time course using direct culture and PCR

3. Determine detectability and accumulation efficiency of *C. jejuni* by freshwater mussels over a time course using direct culture and PCR

4. Compare detectability of *C. jejuni* in water, with detectability in freshwater mussels in order to assess the suitability of mussels as bio-indicators

5. Determine whether estuarine snails are capable of accumulating *C. jejuni* to concentrations that are detectable by direct culture and PCR

3.2 Materials and Methods

3.2.1 DNA purification from *C. jejuni* seeded samples

In order to detect *C. jejuni* by PCR, a rapid, robust and sensitive method for obtaining DNA template was required. Two methods were tested:

3.2.1.1 DNA extraction from filter membranes

Volumes of 249ml of sterile dH_2O were seeded with 1×10^7 cfu of *C. jejuni* F38011. Seeded water was passed through a 0.45µm pore size cellulose nitrate filter. The filter membrane was aseptically transferred to a sterile universal bottle (20ml) containing 1ml of SET buffer (appendix II) and vortexed thoroughly to dislodge cells from the membrane. DNA was subsequently extracted using the protocol described in section 4.2.2.1. Filter membranes were removed from universal bottles following the cell lysis step.

3.2.1.2 Direct DNA extraction from seeded water and broth

Volumes of 249ml of sterile dH_2O were seeded with 1ml of a 1×10^7 cfu/ml stock of *C. jejuni* F38011. Volumes of 99.6ml of Exeter enrichment broth were seeded with 400µl of a 1×10^7 cfu/ml stock of *C. jejuni* F38011. Samples of 100µl, 500µl and 1ml were immediately taken from seeded broth or water and heat treated for 15min followed by phenol/chloroform extraction (see section 3.2.5 below).

3.2.1.3 Detection limits of DNA extraction from filter membranes

To determine the limitations of the DNA extraction method from filter membranes, 249ml water samples were seeded with 1ml of ten-fold serial dilutions of a 3.5×10^6 cfu/ml stock of *C. jejuni* KLC 4235. Samples were filtered through a 0.45µm filter membrane and DNA extracted as described in section 3.2.1.1.

3.2.1.4 Detection limits of DNA extraction from enrichment broths

Whirl-pac bags containing 99ml of Exeter enrichment broth (minus supplement B) were artificially seeded with 1ml of ten-fold serial dilutions of a 1.733×10^9 cfu/ml stock of *C. jejuni* KLC4235. Samples were subjected to the enrichment conditions

described in section 2.2.8.3. Following enrichment, 10ml aliquots of broth were treated with heat and extracted with phenol/chloroform as described in section 3.2.5.

3.2.2 Polymerase chain reaction (PCR) amplification of the *flaA* gene

In order to assess the PCR detection limits of DNA extracted from filter membranes and enrichment broths, a 458bp internal fragment of the flagellin-A (*flaA*) gene was amplified. PCR reactions were prepared as described in section 4.2.4.1 using the primers pg50 and pg3 at a final concentration of 5pmol per reaction. Amplification consisted of 32 three step cycles (cycle 1: 94°C for 3min, 37°C for 1min and 72°C for 1min, cycles 2-31: 94°C for 1min, 37°C for 1min and 72°C for 1min. Cycle 32 was a final soak step at 4°C to refrigerate reactions until they could be processed). PCR amplicons were resolved by electrophoresis through an 1.5% agarose gel and visualised as described in section 4.2.5.

3.2.3 Detection and survival of *C. jejuni* from freshwater

In order to establish whether aquatic invertebrates are good bio-indicators, it was necessary to determine survival and detectability of *C. jejuni* in water over time.

3.2.3.1 Tank preparation

Detection experiments were performed in PVC tanks. Tanks were cleaned thoroughly by scrubbing in hot water supplemented with pyroneg detergent, Jif and abrasive cleaner (Ajax) followed by extensive rinsing. Each tank was subsequently surface sterilised with 70% ethanol and allowed to air dry. Once dry, tanks were placed into a laminar flow cabinet and exposed to UV light for 30-60min.

3.2.3.2 Experimental set-up

A 25L PVC tank was placed inside a larger 50L tank (due to the containment requirements of the Department of Zoology) and filled with 20L of fresh tap water. Tanks containing water were placed inside a 15°C temperature controlled growth room and covered with plastic lids to prevent losses from evaporation. The growth room has a 6am to 6pm 12h visible light cycle.

3.2.3.3 C. jejuni inoculation

C. jejuni KLC4235 was used as the test strain for these experiments. This particular strain was chosen because it is tetracycline resistant (Tet^R), allowing for specific selection of this strain from samples. Cell growth from 4 CCDA plates previously incubated for 24-48h was suspended in 5ml of PBS and transferred to a sterile universal bottle. *C. jejuni* cfu/ml were quantified as described in section 2.2.7. The entire contents of the *C. jejuni* stock was subsequently used to inoculate a tank containing freshwater.

3.2.3.4 Water analysis

Using sterile 100ml Schott bottles, 4 individual 100ml water samples were taken at hour 0, 1, 2, 4, 8, 12, 24, and 48 post *C. jejuni* inoculation. To ensure reproducibility of results, this experiment was performed in triplicate with sampling extended to include 72 and 96 hour sampling times for subsequent replicates. Each water sample was individually processed as described in section 2.2.9.3; however, the use of muslin cloth for pre-filtration was not required. Cellulose nitrate filters were enriched as described in section 2.2.9.4 Following enrichment, two 100µl aliquots from each broth for each sampling period were spread plated onto CCDA containing Tet and incubated for 24-48h. Typical *Campylobacter* colonies were visualised by Gram stain and confirmed as *C. jejuni* by the sodium hippurate hydrolysis test (see section 5.2.3).

3.2.3.5 Direct culturable C. jejuni counts from water

In order to determine whether cells were surviving or replicating in freshwater tanks, direct culturable counts were taken at each of the sampling times stated above. A small amount of seeded tank water was collected in a sterile universal bottle. This was referred to as the 'stock solution'. Ten-fold serial dilutions were prepared and culturable plate counts performed on post-incubation CCDA (+ Tet) plates (see section 2.2.7) in order to quantify cell numbers at each sampling point.

3.2.4 Detection of *C. jejuni* from mussel flesh

C. jejuni detection from mussel flesh was assessed for comparison with *C. jejuni* detection in water. Clean, ethanol sterilised tanks were set up as stated in section 3.2.3.2 above.

3.2.4.1 Shellfish preparation

Forty individual shellfish were transferred from storage tanks (see section 2.2.12.1) and placed into the experimental tank. Mussels were placed into experimental tanks 24h prior to *C. jejuni* inoculation in order to allow sufficient time for animals to acclimatise to prevailing tank conditions. Following the acclimatisation period, tanks were inoculated with *C. jejuni* KLC4235 as described in section 3.2.3.3. In order to stimulate feeding, an algal suspension (*Chlamydomonas spp.* or *Chlorella vulgaris*) was added to tanks at a concentration of 10ml/L.

3.2.4.2 Shellfish analysis

Four mussels were harvested at hour 0, 1, 2, 4, 8, 12, 24, and 48 post *C. jejuni* inoculation. As with water analysis, this experiment was performed in triplicate with mussel sampling time extended to include 72 and 96 hour harvesting times for successive replicates. Only individuals observed to be actively feeding at time of sampling were removed from the tank.

Each of the four mussels taken at a sampling time were processed as individual samples. Mussels were shucked using sterile implements. Mussel flesh and shellwater was homogenised in a Waring blender using 100ml of Exeter enrichment broth minus supplement B and placed into sterile whirl-pac bags. Broths were enriched as stated in section 2.2.9.3. Aliquots of 100µl of post-incubation broths were spread plated onto CCDA (+ Tet) in duplicate and incubated for 24-48h in a microaerophilic atmosphere. Typical *Campylobacter* colonies on post-incubation plates were Gram stained and confirmed as *C. jejuni* by their ability to hydrolyse sodium hippurate (see section 5.2.3).

3.2.5 Purification of DNA from enrichment broths

For PCR detection of *C. jejuni* from enrichment broths containing invertebrate flesh or filter membranes, DNA template was purified. Aliquots of 10ml of post-incubation enrichment broths were aseptically transferred to sterile 30ml Oak Ridge centrifuge tubes. Broths were centrifuged for 10min, 4°, at 6400 ×g. Supernatants were discarded and pellets resuspended in 500µl of PBS. Suspensions were transferred to sterile 1.5ml Eppendorf tubes and heat-treated by incubating in boiling water for

15min. This treatment is sufficient to lyse any *C. jejuni* cells present. Tubes were allowed to cool for 10min prior to addition of an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). After thorough mixing using a vortex, tubes were centrifuged for 30min, 4°C, at 14800 ×g. The aqueous phase from each tube was transferred to a fresh 1.5ml Eppendorf tube. DNA was precipitated out of solution by adding 2 volumes of 100% ethanol and 0.1 volume 3M sodium acetate followed by gentle inversion of tubes. DNA was harvested by centrifugation for 5min, 4°C, at 2900 ×g. Supernatants were discarded and DNA pellets washed in 70% ethanol. Pellets were subsequently air dried and dissolved in 100µl of ddH_2O containing Rnase (10µg/ml).

Purified DNA was used as template for PCR amplification of the 458bp internal fragment of the *flaA* gene using primers pg50 and pg3 (see section 3.2.2). PCR amplifications were resolved by agarose gel electrophoresis, stained with EtBr and visualised under UV light (see section 4.2.5).

3.2.6 Detection of *C. jejuni* at lower concentrations

To assess the detectability of *C. jejuni* at lower inoculation concentrations, the above set of experiments were repeated using a lower starting concentration of *C. jejuni*. Four 100ml water samples or 4 mussels were sampled at each sampling period as before. However, all 4 water samples or mussels were combined and processed as one sample. This produced one result for each sampling period. Experiments were performed in triplicate as before.

3.2.7 Controls for *C. jejuni* detection experiments

A number of control experiments were performed to eliminate the potential effect of external variables acting to alter true re-isolation rates of the *C. jejuni* test strain.

3.2.7.1 Experimental tanks

Cleaned, ethanol sterilised and UV treated tanks were tested for the presence of residual *Campylobacter*. A sterile cotton swab was dipped in sterile dH_2O and a 10cm × 10cm section of tank swabbed. Cotton swabs were used to streak CCDA plates, which were subsequently incubated for 48h under a reduced O_2 atmosphere. A

second tank swab was used to prepare a whole cell lysate for use as template in a *flaA* PCR. Any bacterial cells present on tank swabs were suspended in 100µl of sterile ddH_2O in a 0.5ml thin-walled PCR tube. Suspensions were heat-treated at 99°C for 10min and cooled to 25°C for 2min. Tubes were centrifuged for 3min at 17400×g to pellet any cellular debris present and the supernatant transferred to a fresh tube. Pellets were discarded. Whole cell lysates were stored at –20°C until required. 10µl of whole cell lysate was used as template in a *flaA* PCR to detect any *Campylobacter* DNA that may have been present in experimental tanks (see section 3.2.2). Only tanks that were negative for *Campylobacter* by both culture and PCR were used for *C. jejuni* detection replicates.

3.2.7.2 Pre-inoculation samples

Prior to *C. jejuni* inoculation of tanks, 2 × 100ml water samples or 2 mussels were harvested to ensure that mussels and water were free of any contaminating *C. jejuni*. Water samples were processed as described in section 3.2.3.4. Mussels were processed as described in section 3.2.4.2. Replicates yielding pre-inoculation samples positive for *Campylobacter* were not considered for analysis and repeated.

3.2.7.3 Efficiency of ethanol sterilisation

Filter holders and the Waring blender were sterilised with 70% ethanol in between processing of individual water and mussel samples respectively. To assess the efficiency of this sterilisation technique, filter holders that previously contained *C. jejuni* were ethanol sterilised and used to filter sterile dH_2O through a cellulose nitrate membrane. Filter membranes were enriched as before and used to culture *Campylobacter* on CCDA plates. A Waring blender flask that previously contained *C. jejuni* was also ethanol sterilised. Sterile Exeter enrichment broth was blended and enriched in an identical manner to mussel flesh enrichments. Enrichments were plated onto CCDA and incubated at 37°C in a microaerophilic atmosphere for 48h.

3.2.7.4 Confirmation of re-isolation of C. jejuni test strain

To confirm that the *C. jejuni* test strain inoculated into water tanks was identical to the *C. jejuni* re-isolated from water and mussel samples, the *flaA* RFLP profiles of *C.*

jejuni isolated at each sampling time was compared with the original RFLP profile of the test strain. *FlaA*-RFLP was performed as described in section 5.2.6.

3.2.8 Inhibitory effect of mussel flesh on *C. jejuni* growth

The effect of mussel flesh on the growth of *C. jejuni* was examined by comparing *C. jejuni* growth on Exeter agar (appendix I) solid medium with growth of *C. jejuni* on Exeter agar containing mussel flesh.

3.2.8.1 Preparation of mussel flesh medium

Two mussels per 100ml of medium were shucked using sterile implements and homogenised to a uniform consistency in a Waring blender in the absence of a diluent. Nutrient broth No. 2 containing bacteriological agar was prepared as described for Exeter agar in appendix I, and sterilised by autoclaving. Media was cooled to 50°C before addition of defibrinated sheep's blood (Gibco-BRL), solution A, supplement B (see table 2.2 for appropriate concentrations), and the mussel flesh homogenate. Media was mixed thoroughly and poured into sterile Petrie dishes within a laminar flow cabinet. Media plates were allowed to dry for 30min.

Exeter agar was prepared as described in appendix I. Exeter agar contained all constituents of mussel flesh agar except the mussel flesh.

3.2.8.2 Inoculation of C. jejuni KLC4235

Growth from a 24-48h culture of the *C. jejuni* test strain on CCDA was collected using 5ml sterile PBS and a glass spreader. Ten-fold serial dilutions of this 'stock' cell suspension were prepared. Aliquots of 100µl from each dilution were spread plated, in triplicate, onto Exeter agar plates and mussel flesh agar plates. Plates were incubated at 37°C in a reduced O_2 atmosphere for 48h. Growth on post-incubation plates of mussel flesh agar and Exeter agar were compared visually and colony counts performed from *C. jejuni* growth on Exeter agar plates.

3.2.9 Accumulation of *C. jejuni* by estuarine snails

As *Campylobacter* could not be detected from environmental sampling of estuarine snails, their ability to accumulate *C. jejuni* from seeded water was assessed.

3.2.9.1 Collection of snails

Snails were collected from the estuary (see section 2.2.9.1.5) immediately prior to use. Only small-medium sized snails (7.1-17.9mm) were selected because these animals tend to be more efficient grazers of bacterial material (I. D. Marsden, personal communication). Snails were collected at low tide when they were completely uncovered.

3.2.9.2 Collection of sediments

The first centimetre of sediment was collected from the snail sampling site and transported back to the laboratory. Sediments were sieved in order to remove any large animals and particles. Sieved sediments were allowed to settle in pasteurised, filter-sterilised estuarine water.

3.2.9.3 Preparation of snails

Snails were rinsed in sterile distilled water to remove sediments and debris from shell surfaces and placed into a container with filter sterilised 'estuarine equivalent water'. Estuarine equivalent water was prepared by diluting 100% seawater (34ppt) to the equivalent salinity of the estuarine water at the snail sampling site (20ppt). The appropriate salinity was confirmed by salinity reading on a hand refractometer. Estuarine equivalent water was pasteurised for 45min at 85°C, cooled to ambient temperature and filtered through a 0.22μm pore size cellulose nitrate filter membrane under a vacuum aspirator. Rinsed snails were kept in estuarine equivalent water for 2h prior to experimental preparation.

3.2.9.4 Experimental set-up

PVC containers of 2L capacity were used for snail accumulation experiments. Containers were cleaned and sterilised as described in section 3.2.3.1. A 1cm deep layer of sieved sediments were placed over the base of experimental containers. Two sediment samples from each container were collected using a sterile cotton swab and individually streaked over the surface of CCDA plates to ensure sediments did not contain culturable *Campylobacter*. Plates were incubated in microaerophilic conditions at 37°C. Containers layered with sediment were filled to capacity with estuarine equivalent water (1.6L per container) and placed into a 50L PVC tank in a

15°C temperature controlled growth room. Approximately 40 snails were placed into each container and allowed a 24h acclimatisation period.

3.2.9.5 Inoculation of C. jejuni

Prior to inoculation of containers with *C. jejuni* test strain KLC4235 (Tet^R), snail samples were taken to ensure they were not already contaminated with *Campylobacter*. Growth from a 24-48h CCDA plate culture of *C. jejuni* KLC4235 was suspended in sterile PBS and inoculated into the water to achieve concentrations of 5.8×10^4 cfu/ml, 5.8×10^2 cfu/ml or 5.8×10^0 cfu/ml per container.

3.2.9.6 Snail analysis

At hour 0, 1, 2, 4, 8, 12, 24, 48, 72, and 96 post *C. jejuni* inoculation, one snail from each container was harvested. This procedure was performed in triplicate with three containers set up for each *C. jejuni* inoculation concentration. Only those snails observed to be active were harvested. Each snail was rinsed in sterile dH_2O to remove sediments and surface sterilised with 70% ethanol to remove any transient *Campylobacter* cells present on shell surface. Snails were placed in separate sterile universal bottles and homogenised in 10ml of Exeter enrichment broth (minus supplement B) using a sterile glass homogenising rod. Snail homogenates were enriched by incubation at 37°C under a reduced O_2 atmosphere for 24-48h following a 2h pre-incubation step. Aliquots of 50µL of post-incubation snail enrichments were spread plated onto CCDA (+ Tet) in duplicate and incubated for 24-48h in microaerophilic conditions. Typical *Campylobacter* colonies on post-incubation plates were Gram stained and confirmed as *C. jejuni* by a positive sodium hippurate hydrolysis test.

For PCR detection of *C. jejuni*, DNA was extracted from broths as described in section 3.2.5. DNA was used as template for PCR amplification of the *flaA* gene using primers pg50 and pg3 as described in section 3.2.2.

3.3 Results

3.3.1 DNA purification from *C. jejuni* seeded water, broth and filter membranes

Campylobacter DNA was successfully purified from filter membranes after passage of 1×10^7 *C. jejuni* F38011 cells. No DNA was obtained from direct samples of seeded water or Exeter enrichment broth using the heat treatment and phenol/chloroform method. However, when 10ml of enrichment broth was centrifuged for 10min, 4°C at 6400 ×g and DNA extraction performed on the resultant pellet, *Campylobacter* DNA was successfully purified as confirmed by PCR amplification of the *flaA* gene. PCR amplification of the 458bp internal fragment of the *flaA* gene using DNA extracted from seeded enrichment broths are shown in figure 3.1.

3.3.2 Detection limits of DNA extraction from filter membranes

The results of the detection limits of DNA extraction from filter membranes are shown in table 3.1.

Table 3.1: Sensitivity of the DNA extraction method from filter membranes

Treatment	Concentration of *C. jejuni* F38011 in 250ml water sample	Purification of DNA Replicate 1	Purification of DNA Replicate 2
1	3.5×10^6 cfu	+[A]	+[A]
2	3.5×10^5 cfu	–	–
3	3.5×10^4 cfu	–	–
4	3.5×10^3 cfu	–	–
5	3.5×10^2 cfu	–	–
6	0	–	–

+ = DNA successfully purified – = DNA not obtained
[A] = *Campylobacter* DNA confirmed by *flaA* PCR amplification using primers pg50 and pg3

These results indicate this method of DNA extraction will only yield PCR-detectable DNA when 3.5×10^6 cells are present on the filter membrane. At lower cell concentrations, no DNA could be PCR amplified suggesting a failure to obtain DNA

from filter membranes. This method does not appear to be sufficiently sensitive for obtaining *Campylobacter* DNA from water at low cell concentrations.

3.3.3 Detection limits of DNA extraction from enrichment broths

The results from the minimum detection limits of *Campylobacter* DNA extracted from enrichment broths are shown in Table 3.2.

Table 3.2: Sensitivity of DNA extraction method from Exeter enrichment broth

Treatment	Initial inocula of *C. jejuni* in 100ml seeded broth	Purification of DNA Replicate 1	Purification of DNA Replicate 2	Purification of DNA Replicate 3
1	1.733×10^{5} cfu	$+^{A}$	$+^{A}$	$+^{A}$
2	1.733×10^{4} cfu	$+^{A}$	$+^{A}$	$+^{A}$
3	1.733×10^{3} cfu	$+^{A}$	$+^{A}$	$+^{A}$
4	1.733×10^{2} cfu	$+^{A}$	$+^{A}$	$+^{A}$
5	1.733×10^{1} cfu	$+^{A}$	$+^{A}$	$+^{A}$
6	1.733×10^{0} cfu	$+^{A}$	$+^{A}$	$+^{A}$
7	1.733×10^{-1} cfu	$+^{A}$	$+^{A}$	$+^{A}$
8	1.733×10^{-2} cfu	$+^{A}$	$+^{A}$	$+^{A}$
9	1.733×10^{-3} cfu	–	–	–
10	1.733×10^{-4} cfu	–	–	–
11	0	–	–	–

+ = *C. jejuni* DNA successfully purified – = *C. jejuni* DNA not obtained
^{A} = *Campylobacter* DNA confirmed by *flaA* PCR amplification using the primers pg50 and pg3

The purification of *Campylobacter* DNA from post-incubation enrichments was achieved from broths containing an initial inoculum of less than one *C. jejuni* cell. This result was highly reproducible (table 3.2). When compared with the detection limits for DNA extraction from membrane filters (Table 3.1), the broth extraction method has a superior sensitivity for obtaining *Campylobacter* DNA. Figure 3.1 shows *flaA* amplicons generated at each broth concentration. A second amplicon of 275bp is apparent at high inoculation concentrations. The origin of this band is unclear.

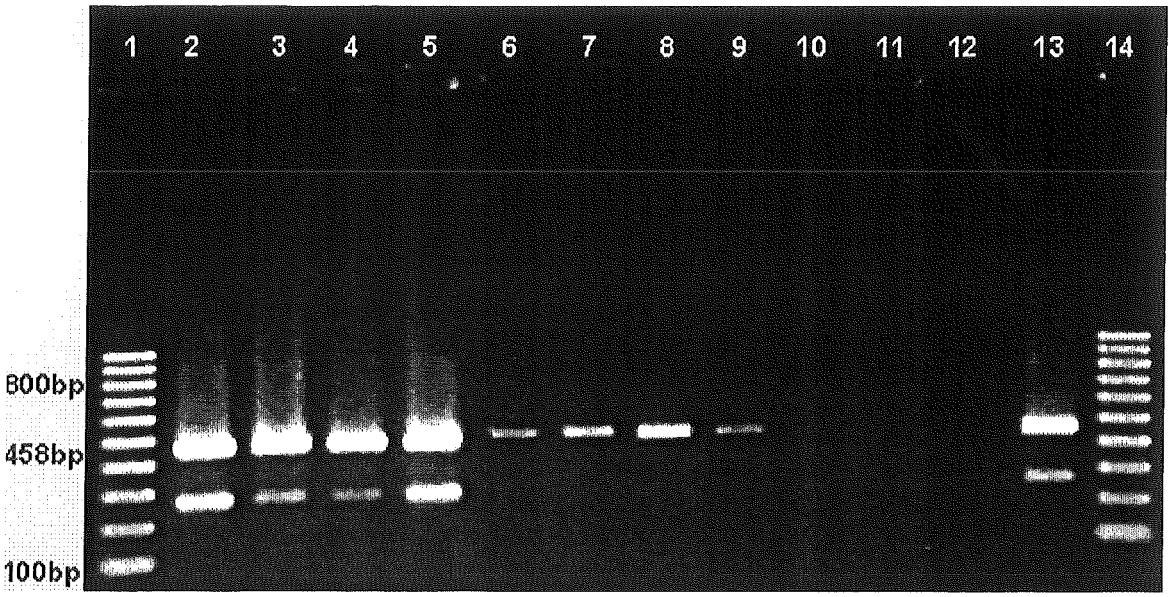

Figure 3.1: Agarose gel of PCR amplicons generated using the *flaA* specific primers pg50 and pg3 with DNA extracted from Exeter enrichment broths seeded with variable concentrations of *C. jejuni* KLC4235 (Replicate 2). Lanes 1and 14, 100bp marker (BioRad); lane 2, DNA from broth seeded with 1.733×10^5 cells; lane 3, DNA from broth seeded with 1.733×10^4 cells; lane 4, DNA from broth seeded with 1.733×10^3 cells; lane 5, DNA from broth seeded with 1.733×10^2 cells; lane 6, DNA from broth seeded with 1.733×10^1 cells; lane 7, DNA from broth seeded with 1.733×10^0 cells; lane 8, DNA from broth seeded with 1.733×10^{-1} cells; lane 9, DNA from broth seeded with 1.733×10^{-2} cells; lane 10, DNA from broth seeded with 1.733×10^{-3} cells; lane 11, DNA from broth seeded with 1.733×10^{-4} cells; lane 12, Negative control (no DNA); lane 13, *C. jejuni* KLC4235 positive control.

3.3.4 Detection of *C. jejuni* KLC4235 from freshwater

The detection of *C. jejuni* KLC4235 in water over time was assessed to provide a base for comparison with invertebrate uptake experiments. The *C. jejuni* KLC4235 inoculum used for each of the water detection replicates and the average over the three replicates is shown in Table 3.3.

Table 3.3: Input concentrations of *C. jejuni* KLC4235 for water detection and mussel accumulation experiments

	Water detection experiments	Mussel accumulation experiments
Replicate 1	2.625×10^4 cfu/ml	2.831×10^4 cfu/ml
Replicate 2	1.615×10^4 cfu/ml	9.511×10^4 cfu/ml
Replicate 3	6.516×10^4 cfu/ml	1.333×10^4 cfu/ml
Average	3.585×10^4 cfu/ml	4.558×10^4 cfu/ml

The number of water samples positive for *C. jejuni* as confirmed by Gram stain, sodium hippurate hydrolysis and *flaA* PCR at each sampling time are indicated in table 3.4.

Table 3.4: Recovery of *C. jejuni* KLC4235 from freshwater over time

Sampling time	Gram stain			Sodium hippurate hydrolysis			*flaA* PCR		
Replicate	**2**	**3**	**4**	**2**	**3**	**4**	**2**	**3**	**4**
Hour 0	4	4	4	2	4	4	4	4	4
Hour 1	4	4	4	2	4	4	4	4	4
Hour 2	4	4	4	4	4	4	4	4	4
Hour 4	4	4	4	4	4	4	4	4	4
Hour 8	4	4	4	4	4	4	4	4	4
Hour 12	4	4	4	4	4	4	4	4	4
Hour 24	4	4	4	4	4	4	4	4	4
Hour 48	4	4	4	4	4	4	4	4	4
Hour 72	4	4	4	4	4	4	4	4	4
Hour 96	2	4	4	2	4	4	3	4	4

0-4 = Number of water samples generating a positive result

Water samples taken prior to inoculation with *C. jejuni* for replicate 1 were positive for *Campylobacter* (data not shown). Consequently, the results from this replicate were not considered for further analysis. A fourth water detection replicate was performed to ensure reproducibility of results with 3 legitimate replicates.

Table 3.4 shows that *C. jejuni* was detectable in 100% of water samples by both direct culture and PCR from hour 0 up to hour 72 post-*C. jejuni* inoculation for all three replicates. At 96 hours post-*C. jejuni* inoculation a combined replicate total of 10 out of 12 (83.3%) of water samples were positive by culture whereas 11 out of 12 (91.6%) of water samples were positive by PCR. Water samples taken at 2 and 3 weeks post-inoculation were negative for *C. jejuni* by both culture and PCR (data not shown). A representative sample of typical *flaA* PCR amplicons generated from water sample enrichments are shown in figure 3.2. Amplicons generated at every sampling interval were identical to those shown below.

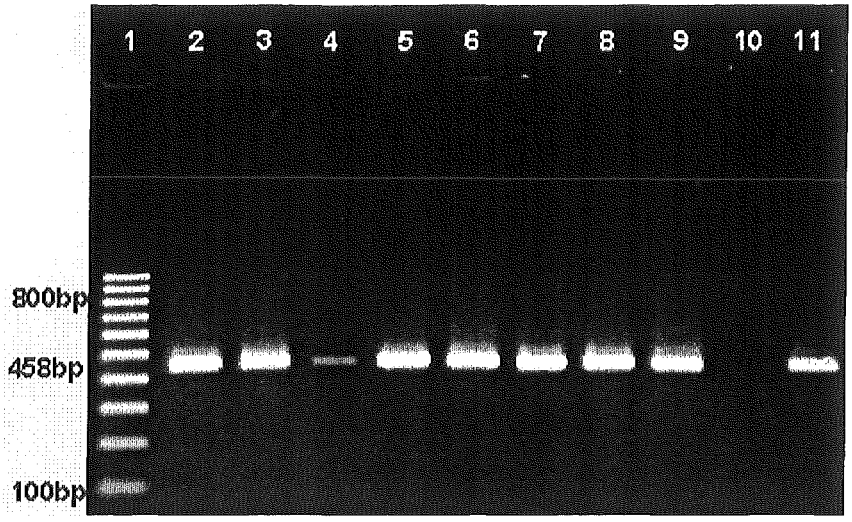

Figure 3.2: Agarose gel of PCR amplicons generated using the *flaA* specific primers pg50 and pg3 with DNA extracted from enrichments of water samples taken at variable time intervals post-inoculation of *C. jejuni* KLC4235 (Replicate 4). lane 1, 100bp marker (BioRad); lane 2, DNA from water sample 1 taken at 24 hours post *C. jejuni* inoculation; lane 3, DNA from water sample 2 taken at 24 hours post *C. jejuni* inoculation; lane 4, DNA from water sample 3 taken at 24 hours post *C. jejuni* inoculation; lane 5, DNA from water sample 4 taken at 24 hours post *C. jejuni* inoculation; lane 6, DNA from water sample 1 taken at 48 hours post *C. jejuni* inoculation; lane 7, DNA from water sample 2 taken at 48 hours post *C. jejuni* inoculation; lane 8, DNA from water sample 3 taken at 48 hours post *C. jejuni* inoculation; lane 9, DNA from water sample 4 taken at 48 hours post *C. jejuni* inoculation; lane 10, Negative control (no DNA); lane 11, *C. jejuni* KLC 4235 positive control

3.3.5 Culturable counts from freshwater

Culturable counts of *C. jejuni* KLC4235 from seeded tank water taken at each sampling period showed cells remained culturable up to the 48 hour sampling time. The colony counts obtained at each sampling time are shown in Table 3.5.

Table 3.5: Culturable *C. jejuni* KLC 4235 counts from seeded freshwater (15°C) over time

Sampling time	Colony counts (cfu/ml) Replicate 2	Colony counts (cfu/ml) Replicate 3	Colony counts (cfu/ml) Replicate 4	Colony counts (cfu/ml) average
Hour 0	2.625×10^4	1.615×10^4	6.516×10^4	3.585×10^4
Hour 1	2.670×10^3	8.260×10^3	1.700×10^4	9.310×10^3
Hour 2	1.750×10^3	5.730×10^3	4.670×10^3	4.050×10^3
Hour 4	5.800×10^2	3.630×10^3	2.860×10^4	1.094×10^4
Hour 8	5.667×10^2	2.930×10^3	4.270×10^3	2.589×10^3
Hour 12	2.033×10^3	8.000×10^3	2.400×10^2	3.424×10^3
Hour 24	1.666×10^2	8.300×10^3	3.670×10^2	2.944×10^3
Hour 48	0*	1.600×10^2	1.330×10^2	9.700×10^1
Hour 72	0*	0*	0*	0*
Hour 96	0*	0*	0*	0*

* = A zero indicates that no *C. jejuni* colonies were present on triplicate plates for any dilution, however, this may not necessarily indicate zero *C. jejuni* cells present in water samples

Figure 3.3 demonstrates the pattern of *C. jejuni* decline as determined by the culturable counts from freshwater. Although *C. jejuni* culturability declines steadily from the time of inoculation to the time when culturability is lost (72h), transient increases in cell numbers are observed at hour 4 and hour 12. This suggests that cells may be capable of multiplying in water, or forming micro-cells through erroneous cell division. However, the general trend is for cell numbers to steadily decline after one hour of being inoculated into the water.

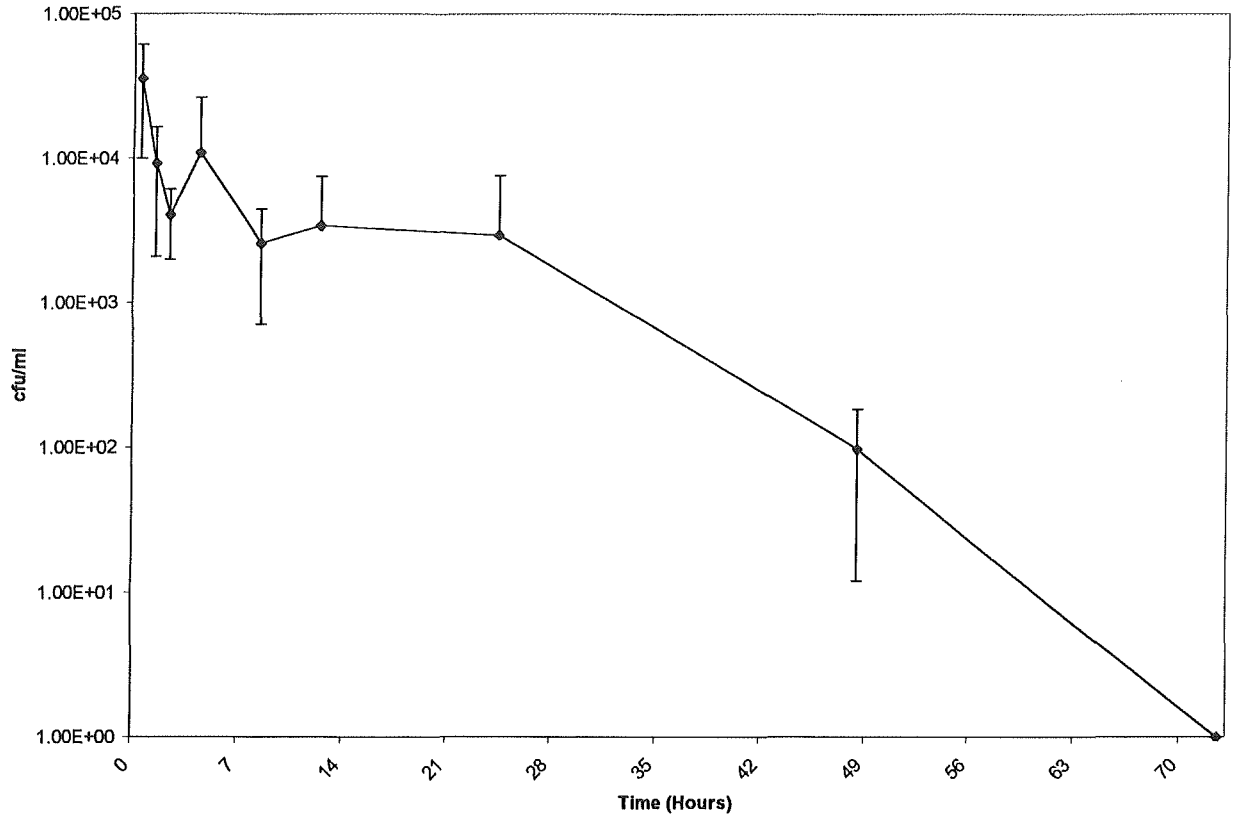

Figure 3.3: Culturable counts of *C. jejuni* KLC 4235 from freshwater over time

3.3.6 Detection of *C. jejuni* from mussel flesh

The accumulation of *C. jejuni* KLC 4235 by the freshwater mussel and its detection from mussel flesh was assessed in order to establish suitability of the mussel as a bio-indicator. The *C. jejuni* KLC 4235 inoculum used for each of the mussel accumulation replicates and the average inocula over the three replicates are shown in Table 3.3. The number of mussels positive for *C. jejuni* KLC4235 at each sampling time, as confirmed by Gram stain, sodium hippurate hydrolysis and *flaA* PCR are indicated in table 3.6.

Table 3.6: Recovery of *C. jejuni* KLC4235 from mussel tissue over time

Sampling time	Gram stain			Sodium hippurate hydrolysis			flaA PCR		
Replicate	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Hour 0	0	4	4	0	4	4	0	4	4
Hour 1	4	4	4	4	4	4	4	4	4
Hour 2	4	4	4	4	4	4	4	4	4
Hour 4	3	4	4	4	4	4	4	4	4
Hour 8	4	4	4	4	4	4	4	4	4
Hour 12	4	4	4	4	4	4	4	4	4
Hour 24	4	4	4	4	4	4	4	4	4
Hour 48	4	4	4	4	4	4	4	4	3
Hour 72	–	4	4	–	4	4	–	4	4
Hour 96	–	4	3	–	4	4	–	4	4

0-4 = number of mussels giving a positive result – = sample not taken at that time period

C. jejuni accumulation by mussels was rapid with positive results obtained immediately (Hour 0) upon *C. jejuni* inoculation of water (for replicates 2 and 3). *Campylobacter* was detected from mussel flesh in 100% of samples by at least one method of detection (direct culture or PCR) from hour 1 up to hour 48. Replicate 1 was performed over a 48 hour time course only. Replicates 2 and 3 were extended to include 72 and 96 hour sampling intervals. At hours 72 and 96, *C. jejuni* was detectable in 100% of mussels by both PCR and direct culture (replicates 2 and 3). Mussels tested at 2 weeks post-inoculation were negative for *C. jejuni* by both direct culture and PCR. One mussel was positive for *C. jejuni* by both culture and PCR at 3 weeks post-inoculation (data not shown). Typical *flaA* PCR amplicons generated from mussel flesh enrichments were identical to those shown in figure 3.2 at every sampling interval.

3.3.7 Detection of *C. jejuni* from water and mussels at lower concentrations

Detection experiments were repeated using a lower initial inoculum of *C. jejuni* KLC 4235. The inoculum concentration of each replicate and the average inoculum is shown in Table 3.7.

Table 3.7: Input concentrations of *C. jejuni* KLC4235 for water detection and mussel accumulation experiments at lower concentrations

	Water detection experiments	Mussel accumulation experiments
Replicate 1	2.321×10^2 cfu/ml	3.421×10^2 cfu/ml
Replicate 2	8.492×10^2 cfu/ml	2.318×10^2 cfu/ml
Replicate 3	2.687×10^2 cfu/ml	7.911×10^2 cfu/ml
Average	4.500×10^2 cfu/ml	4.550×10^2 cfu/ml

The number of mussel samples or water samples positive for *C. jejuni* KLC4235 at each sampling interval as confirmed by direct culture and PCR are shown in table 3.8.

Table 3.8: Recovery of *C. jejuni* KLC4235 from freshwater and mussels at lower concentrations.

Sampling time	Water sampling Gram stain			Mussel accumulation Gram stain			Water sampling hippurate hydrolysis			Mussel accumulation hippurate hydrolysis			Water sampling *flaA* PCR			Mussel accumulation *flaA* PCR		
Replicate	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Hour 0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Hour 1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Hour 2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Hour 4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Hour 8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Hour 12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Hour 24	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Hour 48	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Hour 72	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

+ = Positive *C. jejuni* detection – = *C. jejuni* not detected

As can be seen from Table 3.8, *C. jejuni* was detected by both direct culture and *flaA* PCR in 100% of water and mussel samples from hour 0 up to hour 48 post-*C. jejuni* inoculation for all three replicates. *C. jejuni* was not detectable from water or mussels beyond 48 hours post-inoculation. Even though the concentration of *C. jejuni* per tank was 1% of the original experiments, *C. jejuni* was persistent in water and mussel flesh for 48 hours. The survival of *C. jejuni* in water exactly matches *C. jejuni*

detection from mussel flesh for this set of experiments. Typical *flaA* PCR amplicons generated from mussel flesh and water enrichments are shown in figure 3.4.

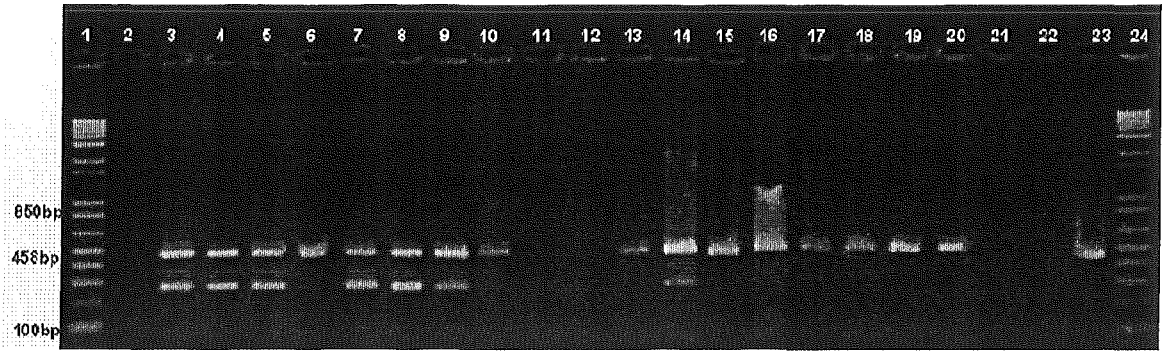

Figure 3.4: Agarose gel of PCR amplicons generated using the *flaA* specific primers pg50 and pg3 with DNA extracted from enrichments of mussels and water taken at variable time intervals post-inoculation of *C. jejuni* KLC4235 (Replicate 2). Lane 1, 1kb plus marker (GibcoBRL); lane 2, Pre-inoculation mussel (negative control); lane 3, DNA from mussel sample taken at 0 hours post *C. jejuni* inoculation; lane 4, DNA from mussel sample taken at 1 hour post *C. jejuni* inoculation; lane 5, DNA from mussel sample taken at 2 hours post *C. jejuni* inoculation; lane 6, DNA from mussel sample taken at 4 hours post *C. jejuni* inoculation; lane 7, DNA from mussel sample taken at 8 hours post *C. jejuni* inoculation; lane 8, DNA from mussel sample taken at 12 hours post *C. jejuni* inoculation; lane 9, DNA from mussel sample taken at 24 hours post *C. jejuni* inoculation; lane 10, DNA from mussel sample taken at 48 hours post *C. jejuni* inoculation; lane 11, DNA from mussel sample taken at 72 hours post *C. jejuni* inoculation; lane 12, Pre-inoculation water sample (negative control); lane 13, DNA from water sample taken at 0 hours post *C. jejuni* inoculation; lane 14, DNA from water sample taken at 1 hour post *C. jejuni* inoculation; lane 15, DNA from water sample taken at 2 hours post *C. jejuni* inoculation; lane 16, DNA from water sample taken at 4 hours post *C. jejuni* inoculation; lane 17, DNA from water sample taken at 8 hours post *C. jejuni* inoculation; lane 18, DNA from water sample taken at 12 hours post *C. jejuni* inoculation; lane 19, DNA from water sample taken at 24 hours post *C. jejuni* inoculation; lane 20, DNA from water sample taken at 48 hours post *C. jejuni* inoculation; lane 21, DNA from water sample taken at 72 hours post *C. jejuni* inoculation; lane22, Negative control (no DNA); lane 23, *C. jejuni* KLC 4235 positive control; lane 24, 1kb plus marker (GibcoBRL)

3.3.8 *C. jejuni* detection controls

3.3.8.1 Efficiency of ethanol sterilisation

No *Campylobacter* was culturable or detectable by PCR from ethanol-sterilised filter holders or the ethanol sterilised blender flask. This confirms the usefulness of ethanol sterilisation when autoclaving is not possible. This result eliminates the possibility of external contamination of samples via processing equipment and thus confirms the presence of *C. jejuni* from the water sample or mussel.

3.3.8.2 Confirmation of re-isolation of original C. jejuni strain

The RFLP patterns generated by restriction (*DdeI*) digestion of the *flaA* gene obtained by PCR performed on representative mussel and water samples showed an identical

profile to the original test strain. This confirms that the *C. jejuni* test strain used to inoculate water was the same strain recovered from mussel and water samples. This result also confirms that the environmental stresses associated with water and mussels did not result in a genetic change in the *flaA* gene of *C. jejuni* KLC4235. The typical RFLP profiles generated by *DdeI* digestion of the *flaA* gene are shown in figure 3.5.

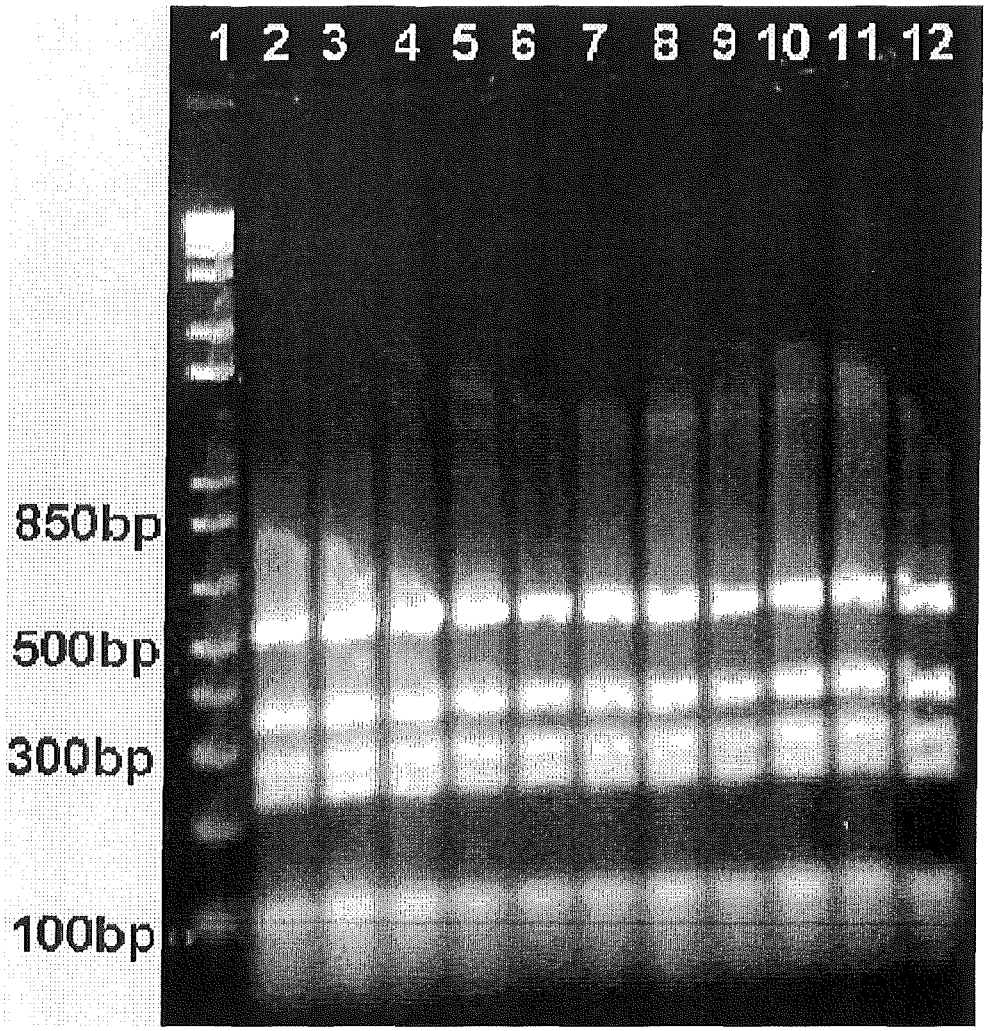

Figure 3.5: Agarose gel of RFLP profiles generated by *DdeI* restriction digestion of the *flaA* gene obtained by PCR amplification using the specific primers pg50 and nr2 with DNA from mussel enrichments. Lane1, 1kb plus marker (GibcoBRL); lane 2, mussel harvested at 0 hours post *C. jejuni* inoculation; lane 3, mussel harvested at 1 hour post *C. jejuni* inoculation; lane 4, mussel harvested at 2 hours post *C. jejuni* inoculation; lane 5, mussel harvested at 4 hours post *C. jejuni* inoculation; lane 6, mussel harvested at 8 hours post *C. jejuni* inoculation; lane 7, mussel harvested at 12 hours post *C. jejuni* inoculation; lane 8, mussel harvested at 24 hours post *C. jejuni* inoculation; lane 9, mussel harvested at 48 hours post *C. jejuni* inoculation; lane 10, mussel harvested at 72 hours post *C. jejuni* inoculation; lane 11, mussel harvested at 96 hours post *C. jejuni* inoculation; lane 12, *C. jejuni* KLC4235 (input strain)

3.3.9 Inhibitory effects of mussel flesh on growth of *C. jejuni*

To assess whether the mussel flesh itself has an inhibitory effect on the growth of *Campylobacter*, homogenised mussel flesh was incorporated into Exeter agar plates. Colony counts performed on triplicate dilution plates of Exeter agar and mussel flesh agar showed a profound difference in *C. jejuni* growth. Bacterial growth on Exeter agar was quantified as 11.667×10^8 cfu/ml in the original *C. jejuni* stock. When exposed to mussel flesh in agar complete inhibition of *C. jejuni* growth ensued. This was the case in all replicate plates. The complete inhibition of *C. jejuni* growth on mussel flesh agar was surprising given the consistent isolation rates from broths containing mussel flesh over the 96 hour time course. A marked difference between *C. jejuni* growth on solid and liquid media containing mussel flesh is apparent (data not shown).

3.3.10 Detection and accumulation of *C. jejuni* by snails

As *Campylobacter spp.* were not isolated from estuarine snails from environmental sampling, the *C. jejuni* accumulation potential and detection from snails was assessed. *C. jejuni* KLC 4235 inocula used for the snail accumulation experiments and the average inoculum over the three replicates are shown in table 3.9.

Table 3.9: Starting inocula of *C. jejuni* KLC4235 for snail accumulation experiments

Tank	Replicate 1	Replicate 2	Replicate 3	Average
1	8.213×10^4	4.841×10^4	4.448×10^4	5.834×10^4
2	8.213×10^2	4.841×10^2	4.448×10^2	5.834×10^2
3	8.213×10^0	4.841×10^0	4.448×10^0	5.834×10^0

The presence or absence of *C. jejuni* from snail enrichments at each sampling interval is shown in table 3.10

Table 3.10: Recovery of *C. jejuni* KLC4235 from snails over time at different inoculation concentrations

Average inocula	Hour 0	Hour 1	Hour 2	Hour 4	Hour 8	Hour 12	Hour 24	Hour 48	Hour 72	Hour 96
Replicate	1 2 3	1 2 3	1 2 3	1 2 3	1 2 3	1 2 3	1 2 3	1 2 3	1 2 3	1 2 3
Tank 1 5.8×10^4	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	− − −
Tank 2 5.8×10^2	+ + +	− − +	− − −	+ + +	+ + +	+ − +	− − −	− − −	− − −	− − −
Tank 3 5.8×10^0	+ + +	− − −	− − −	+ − +	− − −	− − −	− − −	− − −	− − −	− − −

+ = Positive *C. jejuni* detection by culture (confirmed by Gram stain and sodium hippurate hydrolysis)
− = Negative *C. jejuni* detection by culture

The accumulation of *C. jejuni* by snails was rapid with positive results observed immediately upon inoculation. Tank 1, containing the highest *C. jejuni* inoculum, consistently gave positive results from hour 0 up to hour 72. At lower concentrations, *C. jejuni* culturability is lost within one hour of inoculation but is regained at 4 hours post-inoculation. At low inocula (tanks 2 and 3) *C. jejuni* appears to be effectively degraded by snails resulting in loss of culturability after 12 and 4 hours post inoculation respectively for tanks 2 and 3. The concentration of *C. jejuni* in the tank appears to be significantly associated with detection by culture in snails. The detection of *C. jejuni* from snail enrichments by PCR is shown in figure 3.6

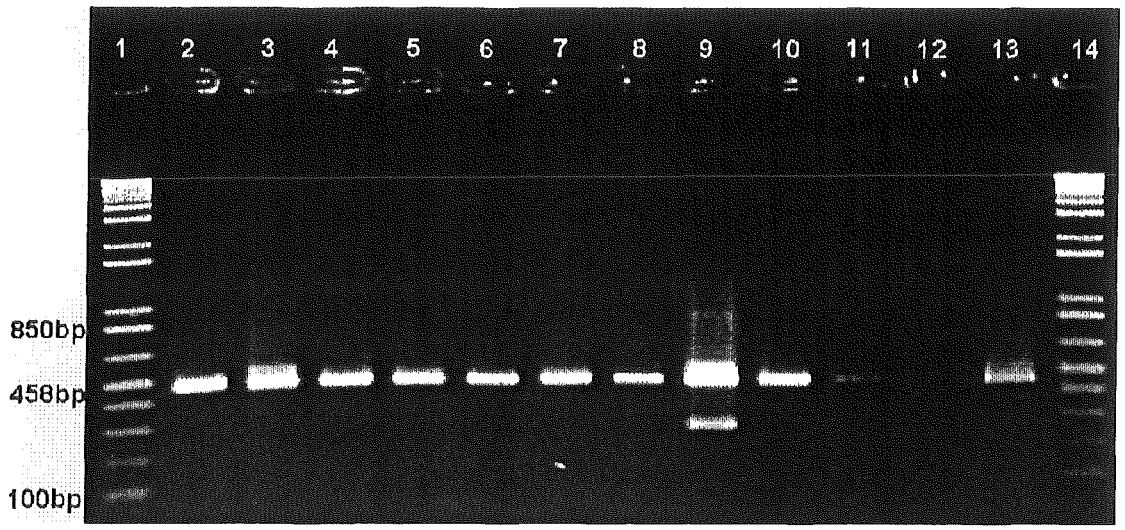

Figure 3.6a: Agarose gel of PCR amplicons generated using the *flaA* specific primers pg50 and pg3 with DNA extracted from enrichments of snails taken from tank 1(5.8×10^4 cfu/ml) at variable time intervals post-inoculation of *C. jejuni* KLC4235 (Replicate 1). Lanes 1 & 14, 1kb plus marker (GibcoBRL); lane 2, DNA from snail sample taken at 0 hours post *C. jejuni* inoculation; lane 3, DNA from snail sample taken at 1 hour post *C. jejuni* inoculation; lane 4, DNA from snail sample taken at 2 hours post *C. jejuni* inoculation; lane 5, DNA from snail sample taken at 4 hours post *C. jejuni* inoculation; lane 6, DNA from snail sample taken at 8 hours post *C. jejuni* inoculation; lane 7, DNA from snail sample taken at 12 hours post *C. jejuni* inoculation; lane 8, DNA from snail sample taken at 24 hours post *C. jejuni* inoculation; lane 9, DNA from snail sample taken at 48 hours post *C. jejuni* inoculation; lane 10, DNA from snail sample taken at 72 hours post *C. jejuni* inoculation; lane 11, DNA from snail sample taken at 96 hours post *C. jejuni* inoculation; lane 12, Negative control (no DNA); lane 13, *C. jejuni* KLC 4235 positive control

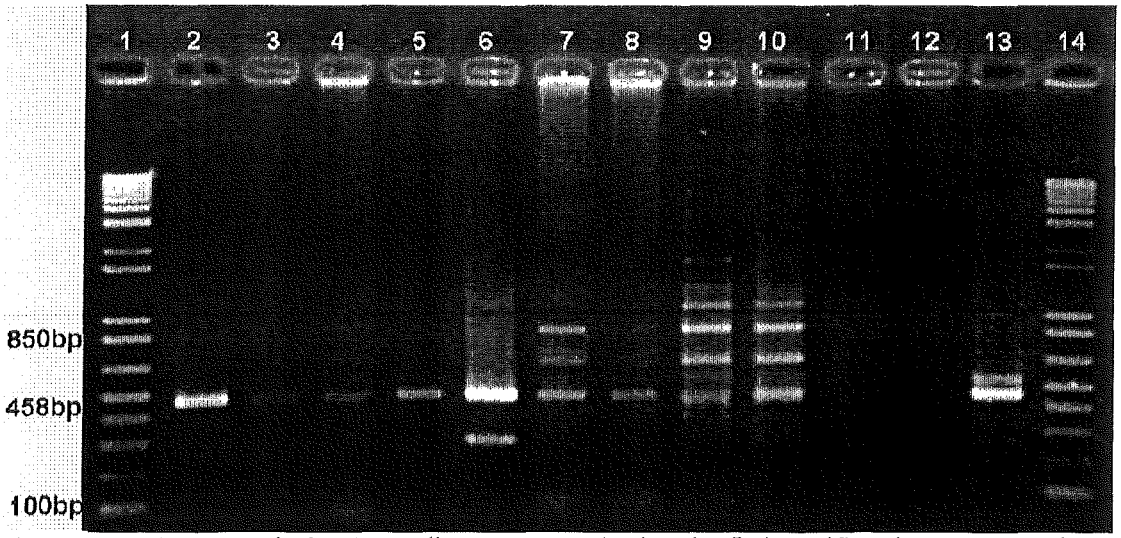

Figure 3.6b: Agarose gel of PCR amplicons generated using the *flaA* specific primers pg50 and pg3 with DNA extracted from enrichments of snails taken from tank 2 (5.8×10^2 cfu/ml) at variable time intervals post-inoculation of *C. jejuni* KLC4235 (Replicate 1). Lanes 1 & 14, 1kb plus marker (GibcoBRL); lane 2, DNA from snail sample taken at 0 hours post *C. jejuni* inoculation; lane 3, DNA from snail sample taken at 1 hour post *C. jejuni* inoculation; lane 4, DNA from snail sample taken at 2 hours post *C. jejuni* inoculation; lane 5, DNA from snail sample taken at 4 hours post *C. jejuni* inoculation; lane 6, DNA from snail sample taken at 8 hours post *C. jejuni* inoculation; lane 7, DNA from snail sample taken at 12 hours post *C. jejuni* inoculation; lane 8, DNA from snail sample taken at 24 hours post *C. jejuni* inoculation; lane 9, DNA from snail sample taken at 48 hours post *C. jejuni* inoculation; lane 10, DNA from snail sample taken at 72 hours post *C. jejuni* inoculation; lane 11, DNA from snail sample taken at 96 hours post *C. jejuni* inoculation; lane 12, Negative control (no DNA); lane 13, *C. jejuni* KLC 4235 positive control

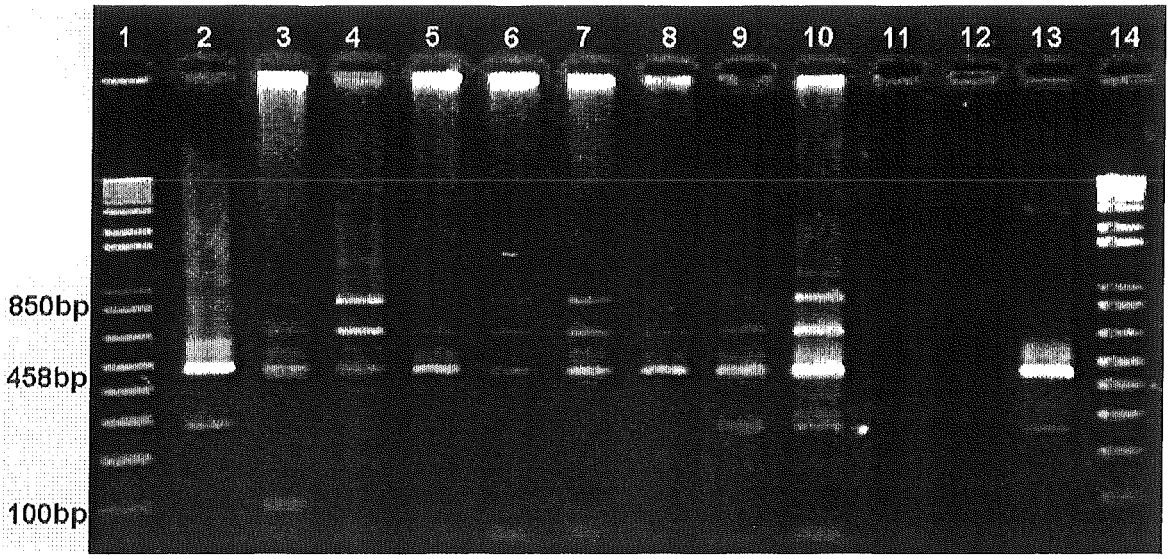

Figure 3.6c: Agarose gel of PCR amplicons generated using the *flaA* specific primers pg50 and pg3 with DNA extracted from enrichments of snails taken from tank 3 (5.8×10^0 cfu/ml) at variable time intervals post-inoculation of *C. jejuni* KLC4235 (Replicate 1). Lane 1, 1kb plus marker (GibcoBRL); lane 2, DNA from snail sample taken at 0 hours post *C. jejuni* inoculation; lane 3, DNA from snail sample taken at 1 hour post *C. jejuni* inoculation; lane 4, DNA from snail sample taken at 2 hours post *C. jejuni* inoculation; lane 5, DNA from snail sample taken at 4 hours post *C. jejuni* inoculation; lane 6, DNA from snail sample taken at 8 hours post *C. jejuni* inoculation; lane 7, DNA from snail sample taken at 12 hours post *C. jejuni* inoculation; lane 8, DNA from snail sample taken at 24 hours post *C. jejuni* inoculation; lane 9, DNA from snail sample taken at 48 hours post *C. jejuni* inoculation; lane 10, DNA from snail sample taken at 72 hours post *C. jejuni* inoculation; lane 11, DNA from snail sample taken at 96 hours post *C. jejuni* inoculation; lane 12, Negative control (no DNA); lane 13, *C. jejuni* KLC 4235 positive control; lane 14, 1kb plus marker (GibcoBRL)

The PCR detection results for *C. jejuni* from snails is in stark contrast to direct culture results. At the highest *C. jejuni* concentration (tank 1), PCR was able to detect *C. jejuni* DNA for the duration of the experiment (96 hours) (fig 3.6a). This compares with culturability of *C. jejuni* up to 72 hours post-inoculation. At lower concentrations, *C. jejuni* detection by PCR is much more sensitive than detection by culture. At an average starting inoculum of 5.8×10^2 cells *C. jejuni* was detectable by PCR up to 72 hours (fig3.6b) from snail enrichments. This compares with culturability of *C. jejuni* for 12 hours. At the lowest average starting inoculum (5.8×10^0 cells), *C. jejuni* is also detectable by PCR for up to 72 hours (fig 3.6c) from snail enrichments while culturability was lost after 4 hours post-inoculation. However, the presence of non-specific bands is increased in PCR products from snail enrichments at lower concentrations (fig 3.6c and 3.6c). The *flaA* band intensity appears to corroborate culturability results. For tanks 2 and 3, culturability was lost at 1 and 2 hours post *C. jejuni* inoculation. The intensity of the PCR amplicon obtained from the

subsequent hour 1 and 2 snail enrichments are significantly less than the hour 4 bands which is the time culturability was re-gained.

3.4 Discussion

3.4.1 Detection limits of DNA purification from seeded samples

DNA extractions were performed using *Campylobacter* cells filtered onto 0.45μm nitrocellulose filter membranes. PCR amplifiable DNA was obtained only from filters that had water containing 3.5×10^6 cells passed through them. These results indicate that this method of DNA extraction is not sufficiently sensitive for PCR detection of *Campylobacter spp.* It is possible that vortexing of filter membranes in SET buffer is not sufficient for dislodging cells from the surface of the membrane into the solution. However, membranes were kept immersed in solution during the lysis of cells with lysozyme and as such, cells that were still adhering to the membrane should also have been lysed. DNA released by lysed cells may have become bound to the membrane and could have been removed along with the filter. This would explain the failure to obtain DNA from water seeded with less than 3.5×10^6 cells. Kirk and Rowe (1994) successfully obtained PCR amplifiable DNA from filter membranes by using sonication to dislodge cells. This method involved filtering *C. jejuni* seeded water samples through a 0.4μm pore size nitrocellulose membrane, sonication for 2min followed by freeze-thaw lysis and heat treatment. A sensitivity of between 10-100 cells per ml was achieved for PCR detection by Kirk and Rowe (1994) using this method. Increasing the intensity of sonication was found to lyse cells and increase the potential for released DNA to bind to the filter. This result further strengthens the possibility of DNA binding to membranes from lysed cells and subsequently being removed along with the filter. Furthermore, Oyofo and Rollins (1993), who tested a range of filter types for obtaining PCR detectable DNA from environmental water samples also reported no amplifiable DNA from nitrocellulose filters. Fluoropore filters were found to be the most effective for obtaining PCR detectable DNA, in this study.

The PCR detection of *Campylobacter* DNA from seeded enrichment broths was found to be significantly more sensitive than from filters. Broths containing an initial inoculum of less than one *C. jejuni* cell yielded PCR detectable DNA. *C. jejuni* cfu/ml was quantified using culturable plate counts. As these counts provide an estimate of the minimum number of cells that may be present in a stock culture, it is

likely that more than the reported <1 *C. jejuni* cell would have been present in these enrichment broths. These cells would have grown to sufficiently detectable levels in post-incubation broths from which DNA was extracted. The use of enrichment broths for extraction of DNA has also been successfully used by a number of researchers. Purdy et al. (1996) used post-centrifugation pellets of *C. jejuni* seeded Preston broth that were suspended in chelex-100 resin and heat treated, for successful PCR amplification. Similarly, Struder et al. (1999) and Hernandez et al (1995) also obtained PCR-detectable DNA from broth cultures using centrifugation and heat treatment.

In the current study, the use of lysates from heat treated Exeter broth pellets did not provide positive PCR results at any cell concentration. This suggested the presence of inhibitors of PCR present in the lysates. Blood (present in Exeter broth cultures) has been shown to inhibit PCR (Purdy et al., 1996) and could have resulted in the lack of PCR amplification products. To remove inhibitors from heat-treated broth lysates, a combination of phenol-chloroform was used to remove proteins and other cell debris not eliminated through lysate preparation. However, aqueous phases obtained from phenol-chloroform extraction and used as template for PCR did not result in amplicons. Therefore, these aqueous phases were used to precipitate DNA from of solution. The use of this purified DNA for PCR resulted in the consistent positive PCR results indicated in Table 3.2. The use of purified DNA for PCR detection of *Campylobacter spp.* appears to be more sensitive than the use of lysates and hence is preferable for efficient *Campylobacter* detection.

3.4.2 *C. jejuni* detection from freshwater

At an average inoculum of 3.585×10^4 cfu/ml, *C. jejuni* KLC 4235 was found to be culturable by enrichments for up to 96 hours in freshwater at 15°C. Water samples taken at two weeks post-*C. jejuni* inoculation were negative for *Campylobacter*. This suggests that *C. jejuni* is culturable in freshwater to some point between 96 hours and two weeks. Temperature, salinity and aeration of water are considered important factors determining the survival of *C. jejuni* in water. Water detection experiments used water at a temperature of 15°C with 0% salinity (freshwater) and minimal aeration. Calder (1998) reported *C. jejuni* culturability in freshwater at a temperature

of 15°C without aeration for a maximum time period of 216h. This is consistent with the 96h-2 week time frame reported for this study. There was little variation in the percentage of water samples positive for *C. jejuni* by culture or PCR. This is indicative of a uniform distribution of *C. jejuni* in the water column. Only at hour 96 was slight reduction in percentage of positive samples evident. At this time period, cell numbers were expected to have declined considerably resulting in the greater variability in isolation rates. Table 3.4 shows that only 2 out of 4 samples were positive for *C. jejuni* by sodium hippurate hydrolysis at hours 0 and 1 for replicate 2. However, Gram stain and PCR confirmed the presence of *C. jejuni*. Negative sodium hippurate hydrolysis results are most likely attributed to concentration effects. For sodium hippurate hydrolysis tests to be accurate, a large loopful of bacterial growth is required. Post-incubation CCDA plates from hour 0 and 1 yielded a bacterial lawn of growth exhibiting typical *Campylobacter* colonial morphology. However, this growth was a relatively thin layer over the surface of the plate making the collection of a loopful of bacterial growth difficult. Furthermore, pre-inoculation water samples taken prior to commencement of the replicate did not reveal any *Campylobacter* contamination. Therefore, it is unlikely that these two samples were contaminated by a *C. coli* strain (as negative sodium hippurate hydrolysis tests are indicative of *C. coli*). *Campylobacter* cells have been reported to become sub-lethally injured upon entering a water source (Mason et al., 1999). This sublethal injury has been associated with a lack of culturability from selective media yet cells remain metabolically active (i.e., retain virulence potential). The use of Exeter enrichment broths for detection of *C. jejuni* from water did not appear to affect *C. jejuni* culturability for the duration of the time course. Even when culturability was lost by direct plate counts, *C. jejuni* was still culturable from enrichment broths (see section 3.4.3 below). This suggests that the 2h pre-incubation period used prior to addition of selective antibiotics was sufficient for allowing sub-lethally injured cells to recover even though pre-incubation times of up to 24h have been suggested (Martin et al, 1996).

3.4.3 Culturable *C. jejuni* counts from water

Culturable counts were taken at each sampling interval for the duration of the water detection time course. The purpose of this experiment was to establish whether *C.*

jejuni cells present in the water were surviving, replicating or in a steady state of decline. *C. jejuni* was found to be detectable by culturable counts up to the 48h sampling interval. At the 72 h time interval, no cells were able to be cultured. This suggests that *C. jejuni* cells present in the water at 15°C are in a steady state of decline. However, transient increases in culturable cell numbers were observed during this 48h time. These increases were consistently observed for each triplicate plate set for all three replicates. However, the specific sampling interval at which increases were observed was variable for each replicate. Table 3.5 shows that an increase in culturable counts was observed at hour 12 for replicate 2 whereas increases were noted at hour 12 and 24 for replicate 3 and hour 4 and 24 for replicate 4. As these increases were consistently reproducible, they cannot be attributed to sampling errors and cell clumping in water samples. Other researchers have also observed these transient increases in colony forming unit counts for *C. jejuni.* Ekweozor et al. (1998) reported transient *C. jejuni* increases in nutrient microcosms of between 0.2 to 2 log units at 4°C. Confirming the results of this study, no predictable pattern in fluctuations of *C. jejuni* culturable counts was observed by Ekweozor et al. (1998). The observed transient increase in culturable cell numbers could be a result of utilisation of dead cells as a nutrient source by remaining culturable cells (Calder, 1998). However, the minimum growth temperature for *C. jejuni* has been reported at 31-32°C (Hazeleger et al., 1998). Therefore, replication in water at 15°C is unlikely. Moreover, freshwater cannot be considered an environment that is sufficiently nutrient rich to promote growth. In contrast to the current study, Ekweozor et al. (1998) did not observe transient increases in nutrient-free microcosms where an unremitting decline in colony counts was apparent. As there was an observed decline of 74% in *C. jejuni* counts within one hour of inoculation of water tanks, it is likely that physiological stress as a result of the physical and chemical conditions of the water resulted in this large proportion of bacteria becoming injured causing cell death (Terzieva and McFeters, 1991). A possible explanation is the phenomenon of micro-cell development in preparation for cell death, where *C. jejuni* cells rapidly increase in length and split unevenly to generate a standard sized cell and a mini-cell. The mini-cells are considered to be genetically incomplete (ie, not complete *Campylobacter* cells) while still remaining culturable for short periods of time preceding death (J. Klena, personal communication).

3.4.4 Accumulation and detection of *C. jejuni* from mussels

The accumulation of *C. jejuni* by freshwater mussels was rapid with *Campylobacter* detectable from actively feeding animals by culture and PCR immediately upon *C. jejuni* inoculation of water. Such rapid uptake of bacterial pathogens has also been observed by Timoney and Abston (1984), Friedl et al. (1992) and Plusquellec et al. (1994). Plusquellec et al. (1994) found mussels to accumulate *Salmonella* to detectable levels by a conventional enrichment method within five minutes of exposure to artificially contaminated water. *Salmonella* was detectable within five minutes of exposure at cell concentrations as low as 3.6 cfu/ml in these mussels. Similarly, Timoney and Abston (1984) reported shellfish to accumulate between 5×10^6 to 1×10^7 cfu of *E. coli* or *Salmonella* in only fifteen minutes of exposure to contaminated water.

In the current study, *C. jejuni* was detectable in 100% of mussels harvested immediately after *C. jejuni* inoculation (Hour 0) in 2 of 3 replicates (Table 3.6). Replicate 1, in which *C. jejuni* accumulation was not observed until one hour post inoculation is most likely a reflection of the health of the mussels used. For replicate 1, a pre-existing stock of mussels (from a previous collection) was used for accumulation experiments. These mussels had been kept in storage tanks without continual feeding for a minimum of six months prior to use and as such the health of these mussels was somewhat compromised. This could have resulted in a longer food stimulation time required for active feeding to commence. Preliminary trials showed that these mussels did commence feeding when algae was used as a stimulant. Mussels used for subsequent replicates were freshly collected and were maintained on regular doses of algae and food supplement (see section 2.2.12.2) resulting in the faster accumulation rates observed.

Time after inoculation of water had little effect on the number of positive samples obtained. At 96h post-inoculation, *C. jejuni* was still detectable in up to 100% of mussels harvested. Given the expected high flow rates of water processed through mussels, it is likely that the entire tank volume of water would have been filtered by each mussel in a relatively short space of time. Assuming that the freshwater mussels used in this study possess a ventilation rate of approximately 1.5 litres per hour as

reported by Boyle (1981), each animal, in just under 13.5h would have filtered the entire contents of the 20L of water present in experimental tanks containing mussels. The fact that *Campylobacter* species remain detectable by both PCR and culture for significantly longer than this amount of time gives rise to a number of possibilities. One such possibility is that the retention capabilities of the freshwater mussel is somewhat lacking in efficiency resulting in *Campylobacter* cells being siphoned in to the animal and directly back out with the water. Given that bivalve molluscs have evolved as specialist filter feeders and particulate matter is a principal food source, this is highly unlikely. Mussels are reported to have the capabilities of retaining particles as small as 1μm (Boyle, 1981). Furthermore, Abad et al (1997) have reported human pathogenic enteric viruses to be accumulated by mussels and retained in the gills and digestive tract. Given the substantial differences in size between bacteria and viruses, mussels should not have any problems retaining bacteria, even though the adherence properties of bacteria and viruses to mussel flesh may differ.

A second possibility is that there is a time delay between accumulation of bacteria and the subsequent processing, digestion and elimination in faeces. A number of studies have addressed the retention of pathogenic bacteria following accumulation by shellfish. Arumagaswamy et al. (1988) reported that oysters that had accumulated *C. jejuni* retained 40-50% of cells initially ingested after 48h, with 4% retention of initial load by 8 days. Friedl et al. (1992) found microbe-size abiotic particles persisted in oysters at a diminishing rate for the 72h duration of their study. In light of the evidence for retention of bacteria in shellfish, it is most probable that accumulated, but undigested cells remain viable and culturable to a readily detectable level for some period of time after cells have been cleared from the water. Mussels tested at 2 weeks post *C. jejuni* inoculation were all negative for *Campylobacter* by both detection methods used. In contrast, one mussel out of four harvested at three weeks was positive for *C. jejuni* by both culture and PCR. It is possible that some *C. jejuni* KLC 4235 cells may have become adapted for survival in water and/or shellfish resulting in such a long persistence time. Given that the data for *C. jejuni* survival and detection in water showed a significant decline in cell numbers ultimately resulting in non-detectability, this is an unlikely notion. An alternative explanation for persistence of *C. jejuni* a mussel for three weeks could be that the animal was no longer

metabolically efficient and accumulated cells were persisting in digestive tissues or shellwater. Although mussels tend to survive for relatively long periods of time, a small percentage of mussels in storage tanks were observed to be dead or dying as indicated by weak adductor mussel contraction (shell closing) and flesh in a visible state of decay. This particular mussel may have been in an early stage of mussel death. In addition, Roper and Hickey (1995) suggested that in low-food situations not unlike those present in experimental tanks at 3 weeks post-inoculation, mussels pump larger volumes of water to obtain food. In these situations, the uptake of bacterial contaminants could be enhanced. Increased pumping rates may have resulted in residual *C. jejuni* cells present in the water being accumulated to a level that was detectable by enrichments.

PCR detection of *C. jejuni* from Exeter enrichment broths containing mussel flesh was particularly effective. *C. jejuni* was detectable by PCR in almost all occasions when *C. jejuni* was detectable by culture. When *C. jejuni* was not detectable by culture, PCR detection results were also negative. This close correlation of PCR detectability with culturability is most likely a reflection of the methodology used. As enrichment broths are used to culture cells present in low numbers in an environmental sample to high numbers in post-incubation broths, it is not surprising that PCR results match that of culture results. Any *C. jejuni* cells that may have entered a VNC state in water or shellfish would not have grown in broth cultures and hence a particularly large number of VNC cells would have to be present in broths in the absence of culturable cells for PCR to solely detect VNC cells. The use of PCR to detect potentially VNC cells using direct samples of water or mussel flesh may have detected the presence these cells. However, the use of PCR for detection of VNC cells has limited use with no real way to differentiate between DNA detected from a VNC cell and DNA detected from a dead cell (Calder, 1998).

3.4.5 *C. jejuni* detection from mussels and water at lower cell concentrations

Water detection and mussel accumulation experiments were repeated using approximately 1% of the original starting *C. jejuni* inoculum. As there was little or no variability in the number of water samples or mussels positive for *C. jejuni* at each sampling interval, all four mussels or 400ml water samples were processed as one

sample. *C. jejuni* was detectable at every sampling interval for each replicate up to 48h in mussels and water. At higher inoculation concentrations, *C. jejuni* was detectable for longer times. From these results, it is apparent that the concentration of *C. jejuni* cells present in the water has a direct effect on the length of time *C. jejuni* can be detected from mussels and water. The effect of concentration and length of detectability does not appear to be directly proportional. At a concentration of approximately 4×10^4 cfu/ml *C. jejuni* was detectable for at least 96h. At 1% of this concentration (4×10^2 cfu/ml), *C. jejuni* was detectable for approximately half this time. This result is in agreement with Plusquellec et al. (1994) who found a low level of variability in detection times for *Salmonella* in water and mussels at different concentrations. The use of a bacterial concentration ten times that of the lowest inoculum resulted in detectability for the same length of time in this study by Plusquellec et al. (1994). Calder (1998) observed *C. jejuni* to be detectable in mussels for 48h at a starting concentration of 4.5×10^6 cfu/ml in tank water. In agreement with the results of the current study, mussels were positive for *C. jejuni* at less than 1% of this concentration (6.9×10^3) for half the length of time (24h). However, this was not consistent over all inoculation concentrations.

3.4.6 Confirmation of re-isolation of *C. jejuni* KLC4235

The *flaA* gene encoding the production of the flagellin has been shown to possess significant sequence heterogeneity, with sequence data indicating substantial divergence between strains (Nachamkin et al., 1993; Owen et al., 1994). To confirm that the *C. jejuni* test strain used to inoculate experimental tanks was identical to the *C. jejuni* re-isolated from water and mussels, the genetic profiles of the *flaA* gene of *C. jejuni* KLC4235 test strain was compared with representative isolates obtained at each time interval from water and mussel samples. Identical profiles of *DdeI* digested *flaA* PCR amplicons were obtained confirming that the test strain was the same strain re-isolated from the water and mussel samples. The *flaA* gene has been considered a relatively unstable region of DNA subject to significant sequence variation (Harrington et al., 1997; Wassenaar et al., 1991; Wassenaar et al., 1995). Environmental stresses such as those present in water and mussels have been implicated, in part, with this genetic variation. Fig 3.5 confirms that passaging *C.*

jejuni through mussels and water has not been responsible for a genetic change in the flagellin gene of *C. jejuni* KLC4235.

3.4.7 Inhibitory effects of mussel flesh

When *C. jejuni* KLC4235 growth on Exeter agar plates was compared to growth on Exeter agar containing mussel flesh, complete inhibition of *C. jejuni* growth was observed on mussel flesh agar plates. This result was unexpected as broth cultures containing mussel flesh were highly effective for *C. jejuni* isolation. A similar result was obtained by Calder (1998) who reported no inhibition of the *C. jejuni* test strain in enrichment broths containing mussel flesh yet a significant reduction of *C. jejuni* on solid media containing shellfish flesh. Although shellfish flesh agar was inhibitory to *C. jejuni* growth, a complete lack of *C. jejuni* growth was not observed by Calder (1998). Interestingly, live marine mussels used by Calder (1998) were found to yield detectable *C. jejuni* for only 48h whereas live freshwater mussels used in this study gave significantly longer *C. jejuni* detection times. This would suggest that freshwater mussels would be less inhibitory to *C. jejuni* than marine mussels. However, differences in ability to eliminate bacterial cells could also explain this difference in detection time frames. It is obvious from these results that substances present in mussel flesh substantially inhibit *C. jejuni* growth. This inhibition is less effective in broth culture however, suggesting differences in the growth conditions present in solid and liquid media have a significant effect of culturability. This may be due to the inhibitory mussel flesh being chelated in broth while being concentrated on the agar

3.4.8 Is the freshwater mussel a good bio-indicator?

Mussel accumulation and water detection experiments were designed to assess the suitability of the mussel as a biological indicator for detection of *C. jejuni* in recreational water supplies. A number of specific criteria were required to be met.

The first criterion for a potential bio-indicator was the ability to concentrate *Campylobacter* cells to levels that are detectable by routine procedures. Mussel detection experiments have clearly shown that the freshwater mussel is capable of accumulating *C. jejuni* cells from the surrounding water to a level that is readily

detectable by standard culture techniques and PCR-based procedures. Donnison and Ross (1999) have successfully used the identical species of freshwater mussel for isolation of *Campylobacter* and other enteric bacteria from New Zealand rivers. Freshwater mussels placed in mesh cages were found to readily accumulate *Campylobacter* cells naturally present in river sites to detectable levels by conventional enrichment culture methodologies. These results unequivocally demonstrate that freshwater mussels accumulate culturable *Campylobacter* cells from water.

The second criterion required to be met by a candidate biological indicator was the ability to maintain cells in a culturable form for longer periods than in the water. Although the VNC state of *C. jejuni* was not directly assessed in mussel accumulation experiments. the use of enrichments for detecting *C. jejuni* essentially negates the detection of any VNC cells present. Had mussels maintained cells in a culturable state for longer periods than the water, *C. jejuni* would have been routinely isolated from mussels at sampling intervals where water samples would have been negative. This was not the case. This suggests that mussels may not maintain cells in a culturable state for longer periods than water. However, whether or not *C. jejuni* KLC 4235 is actually capable of entering a VNC state in water was not directly addressed.

The third criterion required the accumulation of *Campylobacter* in a transient pattern. When the pathogen is present in the water, it is also required to be present in the mussel. Water detection and mussel uptake experiments showed that *C. jejuni* detection in water was very closely correlated with *C. jejuni* detection in the mussel. Just as pre-inoculation water samples taken at the beginning of each water detection replicate were negative for *C. jejuni*, so were mussels that had been placed in water tanks 24h prior to sampling. Similarly, water samples taken at 2 weeks post *C. jejuni* inoculation were negative for *C. jejuni* just as mussels harvested at 2 weeks post inoculation were negative. Although one mussel taken at 3 weeks post inoculation was positive for *C. jejuni*, this was most likely a minor discrepancy and is not indicative of the general trend observed.

Criterion number four required that persistence of the pathogen in the invertebrate bio-indicator must correlate with its persistence in water. High bacterial loads in the water are expected to correlate with higher bacterial loads in the mussel or a greater number of positive invertebrate samples. Uptake experiments revealed that the number of mussel samples yielding positive detection results were virtually identical to the number of positive water samples for the duration of the experiment. In the natural environment, there is little doubt that the level of bacterial loading in the water would correlate with a higher level loading in the mussel at a magnitude of possibly 6-8 times that of the water (Timoney and Abston, 1984).

The final criterion for a suitable bio-indicator to meet was the prevention of cellular adherence to and invasion of invertebrate flesh resulting in colonisation and infection. *C. jejuni* accumulation tests demonstrated that at 2-3 weeks post *C. jejuni* inoculation, the majority (88%) of mussels tested were negative for *Campylobacter*. In addition, the elimination of non-digestible bacterial cells in faeces and pseudofaeces as well as the presence of immune responses such as the production of haemocyte and tissue phagocyte ensure that bacterial colonisation does not occur (Friedl et al., 1992). Overall, the freshwater mussel was found to have met 4 out of 5 of the initial criteria set down for a suitable bio-indicator. Furthermore, the detection of *C. jejuni* in water was found to very closely correlate with detection in the mussel. However, the results obtained suggest that although the detection of *C. jejuni* from mussels was as good as detection from water, it was no better than testing the water itself (by enrichment methods) in terms of isolation rates, simplicity of methodology and time taken to obtain definitive results. Therefore, while the use of the freshwater mussel as a bio-indicator for *Campylobacter spp.* may be a useful adjunct to testing water implicated in disease outbreaks and sporadic cases of campylobacteriosis when isolating the responsible organism proves difficult, under normal circumstances enrichment of water is adequate.

3.4.9 Snail accumulation of *C. jejuni*

The interactions between estuarine snails and *Campylobacter* species have not been previously not been studied. To assess whether the estuarine mudflat snail is capable of accumulating *Campylobacter* cells present in the water column as well as those that

sink to the sediments, *C. jejuni* accumulation experiments were performed. Similar to mussels, snails were observed to accumulate *C. jejuni* immediately upon inoculation of water. The detection of *C. jejuni* by culture was found to be highly dependent on the concentration of *C. jejuni* in the tank (Table 3.10). Interestingly, culturability of *C. jejuni* was lost after one hour of *C. jejuni* inoculation at tank concentrations of 5.8×10^2 and less. This loss of culturability remained for 4 hours at which time culturability was regained. As only active snails were harvested, it is assumed that all were feeding when sampled. Therefore, the possibility that snails were not feeding at these times is unlikely (although it was unclear whether activity equated to feeding). It is conceivable that the loss of culturability at hours 1 and 2 represent the initial decline of *C. jejuni* cells observed previously upon inoculation into the water (Fig 3.3). Similarly the subsequent re-gain in cell culturability in snails could be indicative of the transient increases in cell counts observed in water (Ekweozor et al., 1998). In support of this theory, Harvey et al. (1996) suggested that *C. jejuni* cells present in batch culture systems, although showing a decline in culturability, may still retain some metabolic activity or viability. After a period of adaptation these cells may return to a culturable form. Assuming this holds true for tanks containing snails, the period of adaptation can be considered to be between 1 and 4 hours post-inoculation at which time cells become culturable again. Snails appear to be particularly efficient at eliminating viable *C. jejuni* from their digestive tracts at the cell concentrations present in tanks 2 and 3. Boyle (1981) reported that mud snails found on muddy estuarine shores ingest detritus but digest principally micro-organisms that are abundant in this habitat. Therefore, it is possible that *C. jejuni* cells that sink to the sediments are readily ingested by the snails and efficiently degraded. Marine snails are generally grazers of particulate matter and are equipped with a precise radula (feeding apparatus) for grazing on algal growths and other food particles by a 'licking' motion of the radula (Boyle, 1981). At the highest average cell concentration (5.8×10^4 cfu/ml) snails were consistently positive for *C. jejuni* for up to 72h. This compares with positive samples being obtained for 12h post-inoculation at 5.8×10^2 cfu/ml and only 4h at 5.8×10^0 cfu/ml. These low detection time spans may be attributed to the effects of salinity on the survival of *C. jejuni* in water. Salinities of greater than 1% have been demonstrated to be bactericidal to *C. jejuni* (The National Advisory Committee on Microbiological Criteria for Foods, 1995). In

contrast, Calder (1998) found that water of a salinity equivalent to that of estuarine water (18-20ppt) resulted in the longest period of persistence when compared to freshwater and seawater. These conflicting reports may be due to strain-specific effects. The potential toxic effects of salinity and the efficient digestion of bacterial cells by snails may have resulted in the lack of *C. jejuni* isolation from environmental samples of snails and estuarine water.

The use of PCR for the detection of *C. jejuni* DNA from snail enrichments was significantly different to culturability results. PCR detection of *C. jejuni* showed cells to persist for considerably longer than culturability was maintained. A possible explanation for this variation could be that the presence of snail flesh in enrichment broths retards the growth of *C. jejuni* populations, however, PCR is sufficiently sensitive for detection of these cells. PCR detection of *C. jejuni* at lower cell concentrations resulted in a considerable amount of non-specific DNA present in amplification products (fig3.6c). At these low *C. jejuni* concentrations, any competitive inhibition effects *C. jejuni* may be having on snail microflora would be decreased and hence these non-target organisms may be growing to higher numbers in broths with less *C. jejuni* cells. The 458bp amplicon amongst the non-specific bands cannot be confidently inferred as a *C. jejuni flaA* product and needs to be confirmed by sequencing or Southern hybridisation. The results of snail accumulation experiments show that snails are capable of accumulating *C. jejuni* to detectable levels when present in overlying waters.

Chapter IV

cadF Validation Study

4.1 Introduction

In epidemiological studies, the rapid identification of source bacterial isolates or the accurate detection of pathogens from implicated sources is vitally important for minimising public health risk. Rapid, reproducible and sensitive techniques for pathogen speciation and subspecies determination that are not dependent on growth state or environmental influences are preferred (Scheu et al., 1998). Techniques based on the polymerase chain reaction (PCR) have been used to assess phylogenetic relationships between isolates.

4.1.1 The polymerase chain reaction (PCR)

PCR is a highly sensitive method for the selective identification and detection of specific genomic DNA fragments (Scheu et al., 1998). Since its discovery in the mid 1980's, PCR has been extensively used for numerous applications in molecular biology and medical diagnostics (Scheu at al., 1998). The PCR technique consists of a series of amplification cycles, each comprising three specific steps: 1. denaturation of double stranded DNA to single stranded DNA, 2. annealing of oligonucleotide primers to specific targets within the single DNA strands, and 3. primer extension for synthesis of new DNA strands facilitated by a thermostable DNA polymerase. Each step requires specific temperatures to permit specificity of reaction. Denaturation of DNA utilises elevated temperatures of approximately 92-94°C while primer annealing requires a reduction in temperature to approximately 3-5°C below the melting temperature of the oligonucleotide primers. Annealing temperatures are usually the most variable of the three steps and must be determined empirically to prevent non-specific annealing of primers. For primer extension, the temperature is normally between 72-74°C, allowing

the thermostable *Taq* DNA polymerase to function to its optimum potential. and inactivating any endogenous DNA polymerases which might be associated with the sample. With each amplification cycle, an exponential increase in the number of copies of target DNA is observed (Scheu et al., 1998). PCR amplification products (amplicons) can be detected after agarose gel electrophoresis, by ethidium bromide staining and gel visualisation by UV-light.

4.1.2 Overview of *Campylobacter* PCR-detection systems

The rapid differentiation of *C. jejuni* and *C. coli* from other *Campylobacter spp.* has been the driving force behind the development of many *Campylobacter* PCR-detection systems. As these two species are responsible for almost all cases of campylobacteriosis, their specific detection from stool samples for diagnostic purposes and their detection from environmental samples in epidemiological studies is of considerable importance. A number of specific primers targeting various genes and gene regions have been developed for the specific PCR detection of *Campylobacter* species.

4.1.2.1 flaA

The *flaA* gene encodes flagellin, the major protein subunit of the flagellar filament of *C. jejuni* and *C. coli* (Nuijten et al., 1992). It is one of two highly homologous genes present in a head to tail configuration that encode different flagellin proteins (Wassenaar et al., 1991). The two genes, *flaA and flaB,* are transcribed by separate promoters and both contain approximately 1.7kb showing 95% sequence identity (Fischer and Nachamkin, 1991; Wassenaar et al., 1991). Mutational studies have shown that the product of the *flaA* gene plays an essential role in *Campylobacter* motility and its expression has been implicated as an important factor pertaining to pathogenesis due to *Campylobacter* infections (Guerry et al., 1991; Diker et al., 1992). A number of PCR assays have been derived using specific primers that hybridise to various regions of the *flaA* and to a lesser extent, the *flaB* gene (Wegmuller et al., 1993; Birkenhead et al., 1993; Oyofo et al., 1992). Currently, at least seven *flaA* PCR assays for detection and typing of *Campylobacter spp.* are available (Wassenaar and Newell, 2000). Wegmuller et al., (1993) used a set of primers termed CF03 and CF04 to PCR amplify a region of

intergenic sequence that exists between the *flaA* and *flaB* genes. A total of 27 *C. jejuni* and *C. coli* strains all produced a specific PCR amplicon of 340-380bp in length while 23 related species were negative. A third primer, CF02, was also successfully used in a semi-nested PCR assay to provide amplicons of 180 to 220bp (Wegmuller et al., 1993). These three oligonucleotide primers have subsequently been used to detect *C. jejuni* and *C. coli* directly from surface and drinking water as well as sewage, food (chicken, beef, pork) and environmental samples from chicken farms (Jacob et al., 1996; Kirk and Rowe, 1994; Struder et al., 1998; Waage et al, 1999). Struder et al. (1999) was successful in detecting *C. jejuni* and *C. coli* from sand samples using the CF02 and CF03 primer set even when samples were negative by culture. A major limitation of this PCR system is that it is unable to differentiate between *C. jejuni* and *C. coli* isolates. Furthermore, the lengths of PCR products obtained from different strains vary slightly due to variations in the length of the flagellin intergenic sequence (Wegmuller et al., 1993). This lack of consistency in amplicon size can make the reading of results difficult. This is particularly true when attempting to confirm the identity of PCR products by sequencing or using specific probes which may not hybridise to amplicon size variants.

Birkenhead et al. (1993) used a set of oligonucleotide primers referred to as C1 and C2 for amplification of a 1.3kb fragment of the *flaA* gene. The *flaA* gene comprises a variable region that is flanked by two constant regions. The primer C1 was located in the first constant region of *flaA* while the primer C2 was located in the second constant region (Birkenhead et al., 1993). Using this primer pair, Birkenhead et al. (1993) tested a total of 70 *C. jejuni* isolates and 87 non-*C. jejuni* strains from 26 different genera. All 70 *C. jejuni* strains were reported to yield the 1.3kb PCR amplicon while one *C. coli* isolate also yielded the expected size amplicon. In addition, *C. upsaliensis* produced an 1.7kb amplicon. Although these results appeared to have shown specificity for *C. jejuni*, the fact that *C. coli* produced an identical sized fragment suggests that this system may not accurately differentiate *C. jejuni* from *C. coli*. Oyofo et al. (1992) used an oligonucleotide primer pair termed pg50 and pg3 in a specific PCR detection assay for *C. jejuni* and *C. coli* from clinical specimens. The primer pair pg50 and pg3 were derived from the conserved 5' region of the *flaA* gene of *C. coli* strain VC167. From a total of 47

C. jejuni strains and four *C. coli* strains, 100% of PCR amplifications produced the expected 450bp amplicon. This 450bp amplicon did not arise in over 100 related and non-related strains (Oyofo et al, 1992). Oyofo and Rollins (1993) subsequently used the pg50 and pg3 oligonucleotide primers for successful detection of *C. jejuni* and *C. coli* from environmental water samples. This PCR detection system has also been extensively used for detection of *C. jejuni* from a range of water and invertebrate samples in the current study (Chapter III).

The pg3 reverse primer also has a binding site within the *flaB* gene, in addition to its binding site 450bp downstream of the pg50 hybridisation region in *flaA* (Oyofo and Rollins, 1993). The pg3 binding site on *flaB* of *C. coli* has been reported to be further downstream than in *C. jejuni* and hence three amplicons of 458bp (*C. jejuni* and *C. coli*), 2340bp (*C. jejuni*) and 2363 (*C. coli*) have been observed (Comi et al., 1995). The large species-specific amplicons have not been reported by all researchers however (Oyofo and Rollins, 1993, this study). The use of pg50 with a third oligonucleotide primer (nr2) has also been used to amplify the entire *flaA* gene sequence of approximately 1.73kb (Hernandez et al., 1995). This amplification system has been extensively used in conjunction with PCR-based typing systems (Nachamkin et al., 1993; Nachamkin et al., 1996; and see Chapter V).

4.1.2.2 The 16S rRNA gene

Bacterial 16S rRNA sequences are considered attractive targets for developing molecular diagnostic tests (Cardarelli-Leite et al., 1996). This is because the rRNA is an essential part of ribosomes, is genetically stable and comprises highly conserved as well as variable regions (Giesendorf et al, 1992). PCR primers can be developed to exploit these variable regions which can vary considerably among different bacterial species and hence result in species-specific detection (Giesendorf et al., 1992; Cardarelli-Leite et al., 1996). A vast array of oligonucleotide primers have been used for amplifying *Campylobacter* species-specific regions of the 16S rRNA gene (Vanniasinkam et al., 1999). Giesendorf et al. (1992) used a primer pair targeting the conserved regions of the 16S rRNA gene of *C. jejuni*, *C. coli* and *C. lari*. A 426bp amplicon was achieved in only these three species.

Similarly, Uyttendaele et al. (1994) chose a set of primers termed P1 and P2 for specific detection of *C. jejuni*, *C. coli* and *C. lari*. A 16S rRNA based PCR detection system was also found to be faster and more sensitive for detection of *Campylobacter spp.* in sewage than conventional culturing (Koenraad et al., 1995b).

In addition to *C. jejuni* and *C. coli*, 16S rRNA-based PCR amplification systems have also been used for the detection of less well characterised *Campylobacter* species. Purdy et al. (1996) reported a combination of primers termed P2 and P4 successfully amplified a 603bp DNA fragment in all *Campylobacter spp.* tested except *C. sputorum*. Lawson et al. (1997) were able to detect and differentiate *C. upsaliensis* and *C. helveticus* by 16S rRNA sequences. Primers termed CHCU146F and CU1024R could amplify an 878bp DNA fragment specific to *C. upsaliensis* while the use of a primer pair termed CHCU146F and CH1371R specifically amplified a 1225bp *C. helveticus* sequence. Oyarzabal et al. (1997) reported specific detection of *C. lari* by using a 16S rRNA targeted primer set referred to as CL55 and CL632. The detection of these less commonly encountered *Campylobacter* species is potentially important as they may be associated with enteric disease in humans but are not detected by standard culture methodologies that use highly selective antibiotic-containing media (Lawson et al., 1997). As with the *flaA* gene, 16S rRNA genes have been used successfully on PCR-based typing systems (Marshall et al., 1999).

4.1.2.3 23S rRNA-DNA

Similar to 16S rRNA genes, the 23S rRNA gene shows a mosaic structure of phylogenetically conserved and variable regions (Eyers et al., 1993). Eyers et al. (1993) derived a series of primers that could allow discrimination between thermotolerant *Campylobacter* species. One primer pair termed Therm1 and Therm2 was capable of detecting all thermotolerant *Campylobacter* species. Various combinations of these Therm1 and Therm2 primers with oligonucleotides specific for *C. upsaliensis*, *C. lari*, *C. coli* and *C. jejuni* were claimed to be able to differentiate between each of these species. The reliability and sensitivity of these species-specific primer sets, however, is questionable. More recently, a primer termed Therm4 was used along with the original

Therm1 for highly sensitive detection of all thermotolerant *Campylobacter* species (Fermer and Engvall, 1999). Therm4 was constructed to match perfectly the sequences of these thermotolerant species.

4.1.2.4 Other Campylobacter PCR detection systems

A large number of other genes have been used as PCR targets for identification of *Campylobacter* species. These include a GTP binding protein gene, the *gyrA* and *gyrB* (gyrase) genes, *ceuE* (iron transport protein) gene, the *hip* (hippuricase) gene and a two-component regulator gene (Lawson et al., 1999; Zirnstein et al., 1999; Misawa et al., 1998; Jackson et al., 1996). As the *hip* gene is absent from all *Campylobacter* species except *C. jejuni*, it has the potential to be an ideal PCR-specific target gene for *C. jejuni* (Linton et al., 1997). Using a primer set (HIP400F and HIP1134R) targeting the hippuricase gene, Linton et al. (1997) generated a 735bp PCR amplicon in all strains of *C. jejuni* tested while all *C. coli* and other bacterial strains were negative. As contaminated chicken is considered a major source of *C. jejuni* infections, many PCR-based assays have been used for the detection of *C. jejuni* and *C. coli* from chicken products (Ng et al., 1997; Winters and Slavik, 1995; Hazeleger et al, 1994). Ng et al (1997) found as few as 10cfu/ml of *C. jejuni* in spiked chicken rinses were detectable by a PCR assay, while Winters and Slavik (1995) reported rapid identification of *C. jejuni* in chicken washes using a primer pair referred to as C1 and C4. Although many PCR detection systems for *Campylobacter spp.* are available, few reliably differentiate between the closely related *C. jejuni* and *C. coli*. Multiplex PCR using two or more sets of specific primers may be applicable in these cases.

4.1.3 The *cadF* gene

Adherence to enterocytes has been suggested as one major potential virulence property expressed by pathogenic *Campylobacter* strains (Kelle et al, 1998). When bacteria reach their epithelial target, adherence to the intestinal border may be necessary for establishing active infection (Kelle et al., 1998). A number of adherence factors have been described for *C. jejuni*. These include flagella and the CBF1 and CBF2 (cell binding fraction) proteins (Panigrahi and Bamford, 1996). In 1997, Konkel et al., performed binding

assays that showed specific and saturable binding of fibronectin to *C. jejuni*. Fibronectin is a large, multifunctional glycoprotein that promotes numerous adherence functions in mammalian cells (Konkel et al, 1997). ^{125}I-labelled fibronectin demonstrated specific binding to an outer membrane protein of *C. jejuni* with an apparent molecular mass of 37kDa (Konkel et al., 1997). This outer membrane protein was termed CadF and the gene encoding it *cadF* for *Campylobacter* Adhesin to Fibronectin (Konkel at al., 1997). The open reading frame encoding CadF was found to produce a 326 amino acid protein with 52% similarity to the root adhesin protein of *Pseudomonas fluorescens* (Konkel et al., 1997). Using tissue culture and binding assays, Moser et al. (1997) further confirmed that the CadF adhesin was required for *C. jejuni* adhesion to fibronectin in the human epithelial INT407 cell lines. As adhesion to epithelial cells is important for *Campylobacter* virulence, a PCR assay for detection of pathogenic *C. jejuni* and *C. coli* isolates using the *cadF* gene may be of considerable use.

4.1.4 The *cadF*-PCR detection system

The identification of *Campylobacter* virulence genes in foods, water and infected individuals by PCR is a potentially invaluable tool for determining health risks associated with certain foods and for clinical diagnosis of campylobacteriosis cases (Konkel et al., 1999a). In 1999, Konkel et al., undertook a study to determine whether the *cadF* gene and protein are conserved among *C. jejuni* and *C. coli* isolates and to develop a specific PCR detection assay for these organisms. Using a set of primers termed *cadF*-F38 and *cadF*-R20 that flank the 5' and 3' ends of the *cadF* gene from *C. coli* strain M275, the *cadF* genes from two *C. jejuni* isolates were PCR amplified and sequenced. Based on the sequence alignment of the *cadF* genes from these *C. coli* and *C. jejuni* strains, Konkel et al. (1999a) selected a series of primers and attempted to amplify segments of *cadF* genes from a number of *C. jejuni* isolates. Of all the primer combinations tested, two primers designated *cadF*-F2B and *cadF*-R1B produced a 400bp amplicon in 95% (38 out of 40) of *C. jejuni* isolates tested and 83.3% (5 out of 6) of *C. coli* isolates tested. This equated to 93.5% of all isolates tested positive for the *cadF* product. A small number of related and non-related isolates failed to produce the required 400bp amplicon.

Furthermore, Konkel et al. (1999a) found that several sites within the *cadF* gene sequence appeared to be unique to the *C. coli* M275 *cadF* gene. A second series of primers were tested along with the *cadF*-F2B primer in order to specifically amplify a *C. coli*-only region of the *cadF* gene. Using the *cadF*-F2B primer in conjunction with a primer designated *cadF*-R1C, 5 of 6 *C. coli* isolates amplified a 450bp product while all *C. jejuni* isolates tested were negative. A sensitivity assay using the original *cadF*-F2B and *cadF*-R1B primer pair revealed an amplicon from as few as 100 cells of *C. jejuni*. To assess the efficacy of the *cadF*-F2B/cadF-R1B PCR assay for detecting *Campylobacter* in food products, Konkel et al. (1999a) tested the fluid obtained from 27 plastic wrappings of chickens purchased from grocery stores. A total of 26 of 27 (96.3%) of these store bought chickens tested positive for the 400bp amplicon. Moreover, immunoblot analysis of all *C. jejuni* and *C. coli* isolates using a rabbit anti-37kDa protein serum demonstrated that the CadF protein was conserved in size and antigenicity among the strains tested. Although these results looked promising for the specific detection of *C. jejuni* and *C. coli* strains and differentiation between these strains, the results require further validation.

4.1.5 Objectives for *cadF* validation

The aim of the research described in this chapter is to validate the *cadF*-PCR speciation system proposed by Konkel et al. (1999), by using a range of environmental and clinical *Campylobacter* isolates from New Zealand. Specifically, the objectives are:

1. To assess the discriminatory power of *cadF*-PCR to differentiate between *C. jejuni/C. coli* and other *Campylobacter* species.

2. Demonstrate the ability of the F2B-R1B and F2B-R1C oligonucleotide primer pairs to distinguish *C. jejuni* and *C. coli* isolates.

3. Evaluate the reliability of *cadF*-PCR for determining potential virulence of environmental isolates by testing a number of *C. jejuni* and *C. coli* isolates from both clinical and environmental sources for the presence of the *cadF* virulence gene.

4.2 Materials and Methods

4.2.1 Bacterial strains

The bacterial strains used for *cadF* validation are listed in Table 4.2.

All strains were resuscitated from laboratory stocks stored at –80°C by streak isolation on CCDA containing 3.2mg/L of Cef. Plates containing cells were incubated for 24-48h at 37°C under a reduced oxygen atmosphere.

4.2.2 DNA Purification

Total genomic DNA was obtained by one of two methods:

4.2.2.1 Purification using proteinase K and phenol/chloroform

Cells from 24-48h bacterial cultures were harvested by scraping plates, using 4ml phosphate buffered saline (PBS) and a sterile glass spreader. The resultant cell suspension was transferred to a 30ml Oak Ridge tube and centrifuged for 10min, 4°C, at 6400 ×g. Supernatant was discarded and the pellet re-suspended in 500µl of SET buffer (appendix II). Lysozyme was added to a final concentration of 1.0mg/ml and reactions incubated for 30min at 37°C. After successful cell lysis, 0.1 volume of 10% SDS and a final concentration of proteinase K (0.5mg/ml) was added. The solution was incubated at 55°C for 2h (with occasional inversion). The solution was transferred to a 1.5ml Eppendorf tube before addition of 1/3 volume of 5M sodium chloride. Extraction of the chromosomal DNA was done by addition of 1 volume chloroform prior to centrifugation for 15min, 4°C, at 14800 ×g. The aqueous phase (top layer) was transferred to a fresh 1.5ml Eppendorf tube and extracted by addition of an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) followed by centrifugation for 15min, 4°C, at 14800 ×g. The new aqueous phase was transferred to another 1.5ml Eppendorf tube and genomic DNA was precipitated out of solution by the addition of 2 volumes 100% ethanol and 0.1 volume 3M sodium acetate followed by gentle inversion. DNA was collected by centrifugation for 5min, 4°C, at 2900 ×g and the supernatant discarded. The DNA pellet was washed in 70% ethanol and air dried prior to addition of 100µl of ddH_2O containing Rnase (10µg/ml).

4.2.2.2 Rapid extraction with guanidine thiocyanate

Growth from 24-48hr plates was collected using a sterile cotton swab (Biolab Scientific) and suspended in an 1.5ml Eppendorf tube containing 500µl PBS. Cell suspensions were centrifuged for 1min, 4°C, at 14800×g and the supernatant discarded. The pellet was resuspended in 100µl TE buffer (pH 8.0) prior to mixing with 500µl GES lysis solution (appendix II) and incubation at 60°C for 15min. After successful lysis, determined by the complete clearing of the turbidity inside the tube, 250µl of ice-cold 7.5M ammonium acetate was added and the solution incubated on ice for 10min. The solution was extracted using 500µl of 24:1 chloroform/isoamyl alcohol followed by centrifugation for 13min, 4°C, at 14800 ×g. The aqueous phase was transferred to a new 1.5ml Eppendorf tube and DNA precipitated by addition of 0.5 volume ice-cold isopropanol with gentle inversion. Tubes were incubated at ambient temperature for 10-15min and DNA harvested by centrifuging for 3min, 4°C, at 14800 ×g. The DNA pellet was washed 2-5 times in 70% ethanol, air dried and dissolved in 100µl of ddH_2O with Rnase A (20µg/ml).

4.2.3 Storage of DNA

Purified DNA was initially stored at 4°C for 24-48h to allow the nucleic acid pellet to completely dissolve into the water solution. Purified DNA extracts were subsequently stored at -20°C for the duration of this study.

4.2.4 Polymerase Chain Reaction

PCR primers used throughout this study are listed in Table 4.1.

Table 4.1 PCR primers used throughout this study

Primer	Nucleotide Sequence	Target Gene/Organism
F2B	5'-ttgaaggtaatttagatatg-3'	*cadF-C. jejuni & C. coli*
R1B	5'-ctaatacctaaagttgaaac-3'	*cadF-C. jejuni & C. coli*
R1C	5'-gctacttcttttacttgttc-3'	*cadF-C. coli*
Pg50	5'-atgggatttcgtattaac-3'	*flaA-C. jejuni & C. coli*
Pg3	5'-gaacttgaaccgatttg-3'	*flaA-C. jejuni & C. coli*
Nr2	5'-ctgtagtaatcttaaaacattttg-3'	*flaA-C. jejuni & C. coli*
CiaB-F	5'-caaatttagatgatgcaatgg-3'	*ciaB-C. jejuni & C. coli*
CiaB-R	5'-aattcacaatcttcaagtcc-3'	*ciaB-C. jejuni & C. coli*

4.2.4.1 Preparation of PCR reaction mix

PCR was performed in 0.5ml thin-walled PCR tubes in a total volume of 50µl. Each reaction consisted of the appropriate primers (Table 4.1) at a final concentration of 2pmol per reaction; each dNTP (dATP, dCTP, dGTP, dTTP) at a final concentration of 200µM; $MgCl_2$ added to a final concentration of 2mM; 10% of the total reaction was 10X PCR buffer, 2.5 units of *Taq* DNA polymerase and sterile ddH_2O to bring the reaction volume to 50µl. $MgCl_2$, 10X PCR buffer, dNTPs, and *Taq* polymerase were all obtained from Boehringer Mannheim/Roche. Primers were purchased from Gibco/Life Technologies. Reaction mixes were overlaid with 2 drops of mineral oil (Sigma) and briefly pulsed in a microfuge (MSE micro-centaur).

4.2.4.2 cadF-PCR amplification program

Amplification was carried out in a Corbett Research FTS 320 thermal cycler. Amplification consisted of 32 cycles, three steps per cycle: template denaturation for 1min at 94°C, primer annealing for 1min at 45°C and template elongation for 3min at 72°C. A final soak step at 4°C was included to refrigerate reactions until processing.

4.2.4.3 PCR Optimisation

A series of PCRs were performed to establish the optimal concentrations of $MgCl_2$, dNTPs and oligonucleotide primers in order to determine the ideal amplification rates for the *cadF* product. *C. coli* M275 was used as the test strain for PCR optimisation.

4.2.4.4 Controls

A negative control containing all PCR reagents except DNA template was included in all sets of PCR amplifications for detection of any contamination by extraneous nucleic acid template. DNA from *C. jejuni* F38011 was used as positive control for all F2B-R1B PCRs and *C. coli* M275 for all F2B-R1C amplifications in order to show that the PCR was working as expected.

4.2.5 Agarose gel electrophoresis of PCR products

PCR amplification products were resolved by electrophoresis through 1.5% agarose gels. Gels were constructed by heating the appropriate amount of agarose (ultraPURE; Life Technologies) in 1 x TAE buffer (appendix II) until the agarose dissolved. Gels were cast in perspex gel moulds using plastic combs to form wells. 15µl of PCR product was mixed with 2-3µl of 6 × bromophenol blue gel dye and loaded into each well. 3-5µl of 100bp molecular weight marker (Bio-Rad) was used as a molecular weight reference in all gels to determine whether or not an appropriate PCR amplicon was obtained.

Amplified DNA was separated in a DNA sub cell apparatus (Jordan Scientific Co.) at 100 volts for 1h with the electrical current running from the anode (-) to the cathode (+).

Gels were stained in dH_2O containing 20µl of ethidium bromide (EtBr; 10mg/ml) with gentle shaking for 15-20min and visualised using an Ultra Lum electronic UV transilluminator (254nm). Gels were photographed using a Kodak DC120 'Electrophoresis Documentation and Analysis' digital camera.

4.3 Results

A total of 220 bacterial strains comprising *Campylobacter* isolates from a range of environmental and clinical sources as well as a variety of non-*Campylobacter* isolates were tested using the *cadF* F2B-R1B and F2B-R1C primer sets.

4.3.1 DNA extractions

Two DNA purification methods were used for obtaining nucleic acid template for PCR amplification. Both methods produced DNA of sufficient purity and yield for PCR amplification. Genomic DNA prepared from either *C. jejuni* F38011 or *C. coli* M275 resulted in high quality DNA regardless of the method used. PCR amplicons obtained from DNA extracted using the proteinase K and phenol/chloroform method were marginally more pure than those obtained from DNA using the guanidine thiocyanate method (data not shown).

4.3.2 *cadF* PCR using the F2B-R1B primer set

4.3.2.1 C. jejuni isolates

A total of 134 reported *C. jejuni* isolates were assayed for the *cadF* gene using the F2B-R1B primer pair. Of these isolates, 94 originated from a clinical source (92 isolates from human faeces, 2 isolates from human blood) and 40 were from an environmental source. Environmental *C. jejuni* isolates were from the following sources: seven isolates from water, two from seagulls, 13 from mallard ducks, eight from Canada geese and ten from sheep. The expected 400bp PCR amplicon was obtained from 133 out of 134 (99.25%) of *C. jejuni* isolates tested. The one *C. jejuni* isolate that failed to produce the expected size amplicon was from a Canada goose. Typical *cadF*-F2B-R1B PCR amplicons produced by *C. jejuni* isolates are shown in figure 4.1.

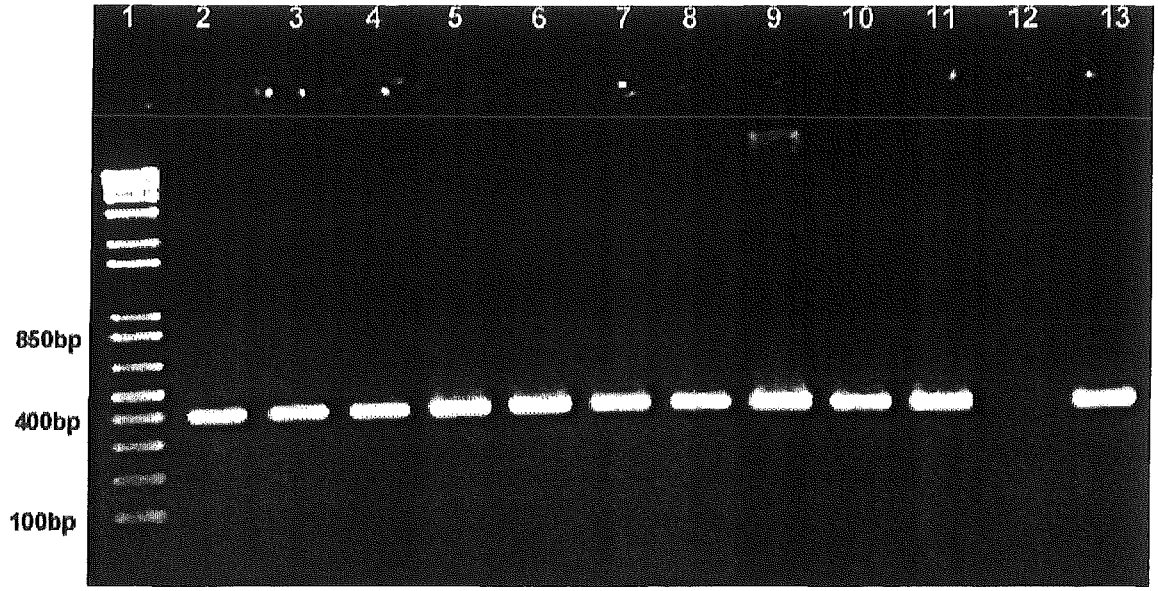

Figure 4.1: Agarose gel of *cadF* PCR amplicons generated using the CadF-F2B-R1B primers with DNA from *C. jejuni* isolates. Lane 1, 1kb plus marker (GibcoBRL); lane 2, ZB632B; lane 3, 776985; lane4, 776736; lane 5, HW961T; lane 6, BN499U; lane 7, 736927; lane 8, 768982; lane 9, 769100; lane 10, 775910; lane 11, 739822; lane 12, Negative control (no DNA); lane 13, F38011 positive control

4.3.2.2 C. coli isolates

A total of 18 *C. coli* isolates were tested for the *cadF* gene. Of these 18 strains, 14 were from a clinical source while four were from a water source. Using the F2B-R1B primer pair, a 400bp PCR amplicon was produced from 17 out of 18 (94.4%) of *C. coli* isolates. The one *C. coli* isolate that failed to generate a 400bp amplicon was a clinical isolate obtained from human faeces.

4.3.2.3 C. lari isolates

Six *Campylobacter* isolates, five of which had previously been presumptively classified as *C. lari* (and the *C. lari* type strain) were tested for presence of *cadF*. A 400bp fragment of DNA was generated from 6 out of 6 (100%) of *C. lari* isolates tested using the F2B-R1B primer set. The type strain of *C. lari* (NCTC11352) generated a number of non-specific bands in addition to the 400bp *cadF* band (data not shown).

4.3.2.4 Other Campylobacter spp.

A total of 13 non-*C. jejuni/C. coli/C. lari Campylobacter* strains were subjected to *cadF* PCR. A 400bp amplicon was generated from three of these 13 (23%) strains. The *Campylobacter* strains positive for the *cadF*-F2B-R1B PCR amplicon were *C. hyoilei*, *C. hyointestinalis* and *C. rectus*. In addition, six of the ten *cadF* negative strains produced non-specific bands. These results are shown in figure 4.2.

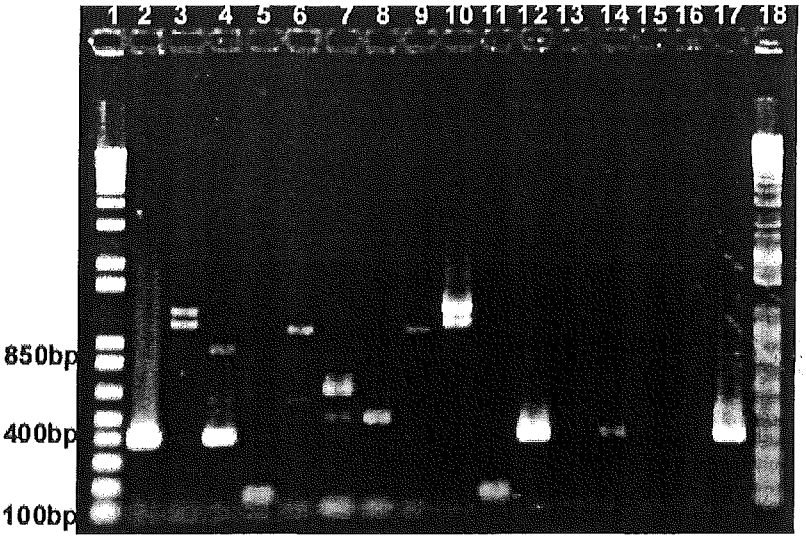

Figure 4.2: Agarose gel of *cadF* PCR amplicons generated using the cadF-F2B-R1B primers with DNA from *Campylobacter* isolates. lane 1, 1kb pus marker (GibcoBRL); lane 2, *C. hyoilei*; lane 3, *C. fetus*; lane 4, *C. hyointestinalis*; lane 5, *C. upsaliensis* (SS0128); lane 6, *C. showae*; lane 7, *C. sputorum subsp. sputorum*; lane 8, *C. sputorum subsp. bulbulus*; lane 9, *C. consisus*; lane 10, *C. fetus subsp. venerealis*; lane 11, *C. upsaliensis* (SS0136); lane 12, *C. jejuni subsp. doylei*; lane 13, *C. gracilis*; lane 14, *C. rectus*; lane 15, *C. curvus*; lane 16, negative control (no DNA); lane 17, *C. jejuni* F38011 positive control; lane 18, 1kb plus marker (GibcoBRL)

4.3.2.5 Non-Campylobacter isolates

A total of 49 non-*Campylobacter* strains were tested for the presence of the *cadF* gene. These isolates comprised a variety of strains closely related and unrelated to *Campylobacter* species. Of the 49 strains tested, two (4%) produced a 400bp amplicon with the F2B-R1B primer pair. The positive results were from *Listeria innocua-B* and *Branhamella catarrhalis,* which produced a faint 400bp band. Of the remaining 47 *cadF* negative strains, three produced unexpected DNA fragments. These were from *Listeria monocytogenes*, *Arcobacter butzleri* and *Arcobacter cryaerophilus*. This result is shown in figure 4.3.

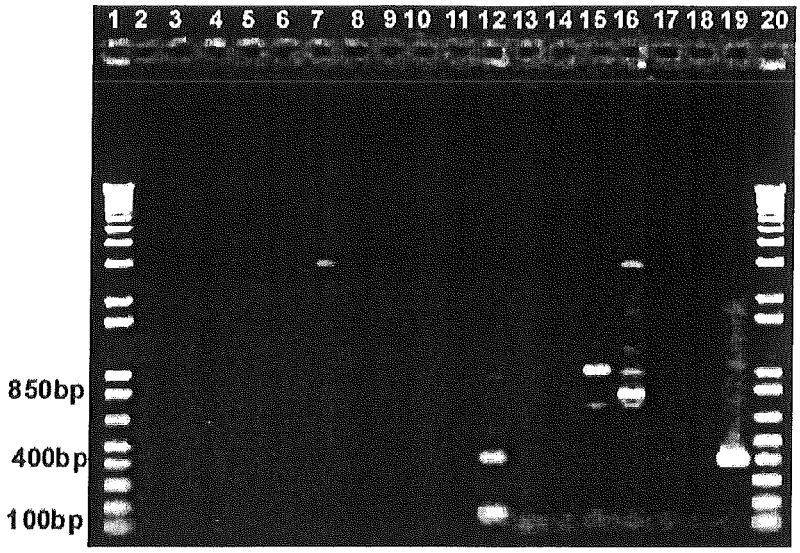

Figure 4.3: Agarose gel of *cadF* PCR amplicons generated using the cadF-F2B-R1B primers with DNA from non-*Campylobacter* isolates. Lane 1, 1kb plus marker (GibcoBRL); lane 2, *Streptococcus bovis*; lane 3, *Proteus vulgaris*; lane 4, *Klebsiella pneumoniae*; lane 5, *Salmonella menston*; lane 6, *Saccharomyces cerevisae*; lane 7, *Listeria monocytogenes*; lane 8, *Listeria ivanovii*; lane 9, *Enterobacter aerogenes*; lane 10, *Staphylococcus epidermidis*; lane 11, *Shigella flexneri*; lane 12, *Listeria innocua-B*; lane 13, *Bacillus cereus*; lane 14, *Helicobacter pylori*; lane 15, *Arcobacter butzleri*; lane 16, *Arcobacter cryaerophilus*; lane 17, *Bacteroides ureolyticus*; lane 18, negative control (no DNA); lane 19, *C. jejuni* F38011 positive control; lane 20, 1kb marker (GibcoBRL)

4.3.3 *cadF*-PCR using the F2B-R1C primer set

4.3.3.1 C. jejuni

Using the *cadF*-F2B-R1C primer pair specific for *C. coli*, one out of 134 (0.7%) of *C. jejuni* isolates produced a 450bp PCR amplicon. This strain (770928) is a clinical isolate obtained from human faeces. Of the 133 *C. jejuni* isolates negative for the *C. coli*-specific *cadF* band, 62 (46.6%) generated non-specific banding patterns with the *cadF*-F2B-R1C primer pair. Modifications in Mg^{2+} concentration in PCR tubes coupled with changes in annealing temperature (raised to 50°C and 55°C) did little to prevent these non-specific bands from appearing. Typical profiles of non-specific bands generated by *C. jejuni* DNA with the *cadF*-F2B-R1C primers are shown in figure 4.4.

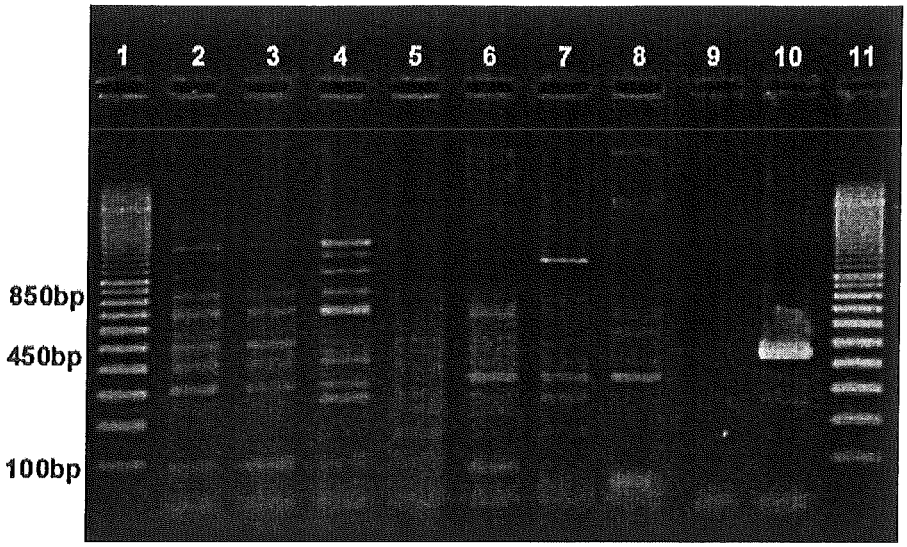

Figure 4.4: Agarose gel of *cadF* PCR amplicons generated using the cadF-F2B-R1C primers with DNA from *C. jejuni* isolates. Lane 1, 100bp marker (GibcoBRL); lane 2, AC013 (duck isolate); lane 3, AC017 (duck isolate); lane 4, AC021 (duck isolate); lane 5, AC058 (goose isolate); lane 6, AC064 (goose isolate); lane 7, AC085 (gull isolate); lane 8, MD672W (clinical isolate); lane 9, negative control (no DNA); lane 10, *C. coli* M275 positive control; lane 11, 100bp marker (GibcoBRL)

4.3.3.2 C. coli

Of the 18 *C. coli* isolates tested for the *C. coli*-specific *cadF*-F2B-R1C PCR amplicon, only 12 (67%) produced the expected 450bp band. Of the 12 positive isolates, all but the M275 control strain routinely produced a second amplicon of approximately 650bp in size. There was no correlation between *C. coli* isolates failing to produce the expected amplicon and the isolate source, with strains from both water and human faeces providing negative results. Typical *cadF* PCR amplicons generated from *C. coli* DNA are shown in figure 4.5.

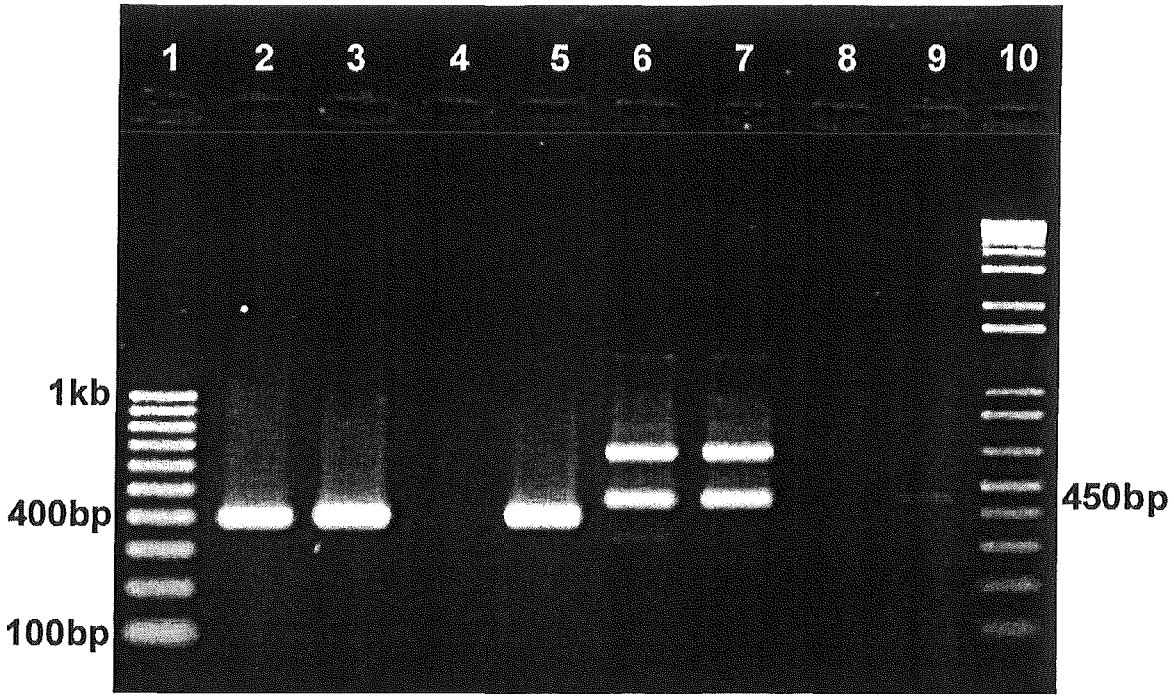

Figure 4.5: Agarose gel of *cadF* PCR amplicons generated using the cadF-F2B-R1B and F2B-R1C primers with DNA from *C. coli* isolates. Lane 1, 100bp marker (BioRad); lane 2, *C. coli* ZP028D (cadF-F2B-R1B amplicon); lane 3, *C. coli* RC167B (cadF-F2B-R1B amplicon); lane 4, negative control (no DNA); lane 5, *C. jejuni* F38011 positive control (cadF-F2B-R1B); lane 6, *C. coli* ZP028D (cadF-F2B-R1C amplicons), lane 7, *C. coli* RC167B (cadF-F2B-R1C amplicons); lane 8, negative control (no DNA); lane 9, *C. coli* M275 positive control (cadF-F2B-R1C); lane 10, 1kb plus marker (GibcoBRL)

4.3.3.3 C. lari

Two out of the six (33%) of *Campylobacter* strains classified as *C. lari* generated a 450bp *cadF*-F2B-R1C amplicon. This included the *C. lari* type strain. All six *C. lari* isolates generated non-specific banding patterns with the *C. coli*-specific primer set (data not shown).

4.3.3.4 Other Campylobacter isolates

Out of the 13 other *Campylobacter* species tested for the *C. coli*-specific band, only *C. hyoilei* generated a 450bp amplicon with the *cadF*-F2B-R1C primers. However, three out of the 12 (33%) of the negative isolates produced non-specific bands. These were from *C. showae*, *C. sputorum subsp. sputorum* and *C. fetus subsp. venerealis* (data not shown).

4.3.3.5 Non-Campylobacter species

From 49 non-*Campylobacter* species tested using the F2B-R1C primers, one strain produced a DNA fragment of 450bp. This was from *Bacillus subtilus* which did not produce a band with the F2B-R1B primers. Four out of the 48 species negative for a 450bp amplicon generated fragments of non-specific DNA. These were from two strains of *Staphylococcus aureus*, *Vibrio parahaemolyticus*, and *Listeria innocua-B*. All of these strains except *Listeria innocua-B* failed to produce an amplicon with the *cadF*-F2B-R1B primers.

4.3.4 Combined *cadF*-PCR results

From a total of 171 *Campylobacter* species tested for the *cadF* gene using the primers F2B and R1B, 159 (93%) gave a positive result. This compares with 2 out of 49 (4%) of non-*Campylobacter* isolates giving a positive result. The individual results of *cadF*-F2B-R1B and F2C-R1C PCR assays for each isolate tested are indicated in Table 4.2.

Table 4.2: Bacterial strains tested for *cadF* validation. + = Expected PCR amplicon generated; - = Expected PCR amplicon not produced; * = non-specific DNA fragments present in PCR products.

Isolate code	Source	CadF-F2B-R1B	CadF-F2B-R1C
C. jejuni			
F38011	Human faeces	+	–
MS961029	Water	+	–
722307	Human faeces	+	–*
726756	Human faeces	+	–*
726757	Human faeces	+	–*
732850	Human faeces	+	–
733034	Human faeces	+	–*
736624	Human faeces	+	–
736927	Human faeces	+	–
739813	Human faeces	+	–
739822	Human faeces	+	–
740081	Human faeces	+	–
740102	Human faeces	+	–*
760624	Human faeces	+	–
760888	Human faeces	+	–
762234	Human faeces	+	–*
762525	Human faeces	+	–
764023	Human faeces	+	–

Table 4.2: continued...

Isolate code	Source	CadF-F2B-R1B	CadF-F2B-R1C
C. jejuni			
768407	Human faeces	+	–
768982	Human faeces	+	–
769100	Human faeces	+	–*
770122	Human faeces	+	–
770608	Human faeces	+	–*
770928	Human faeces	+	+
773635	Human faeces	+	–
773910	Human faeces	+	–
776736	Human faeces	+	–
776985	Human faeces	+	–
780015	Human faeces	+	–
780314	Human faeces	+	–
786483	Human faeces	+	–
787321	Human faeces	+	–
HS019	Human faeces	+	–
WA30	Water	+	–
WA29	Water	+	–*
WA39	Water	+	–*
WA33	Water	+	–*
AC001	Seagull	+	–*
AC003	Mallard Duck	+	–
AC008	Mallard Duck	+	–*
AC011	Mallard Duck	+	–*
AC013	Mallard Duck	+	–*
AC017	Mallard Duck	+	–*
AC021	Mallard Duck	+	–*
AC025	Mallard Duck	+	–
AC029	Mallard Duck	+	–
AC032	Mallard Duck	+	–*
AC034	Mallard Duck	+	–
AC037	Mallard Duck	+	–*
AC039	Mallard Duck	+	–*
AC051	Mallard Duck	+	–*
AC055	Canada Goose	+	–
AC056	Canada Goose	+	–
AC058	Canada Goose	–	–*
AC062	Canada Goose	+	–*
AC064	Canada Goose	+	–*
AC066	Canada Goose	+	–
AC068	Canada Goose	+	–*
AC071	Canada Goose	+	–*
AC073	Water	+	–*
AC079	Water	+	–*
AC085	Seagull	+	–*
ZH614W	Human faeces	+	–
ZI814K	Human faeces	+	–
LT973B	Human faeces	+	–

Table 4.2: continued...

Isolate code	Source	CadF-F2B-R1B	CadF-F2B-R1C
C. jejuni			
LS785M	Human faeces	+	–*
LS897W	Human faeces	+	–*
DO291I	Human faeces	+	–*
DO332T	Human faeces	+	–*
DN031T	Human faeces	+	–*
CI115W	Human faeces	+	–
BB193H	Human faeces	+	–*
XT318V	Human faeces	+	–*
RC317T	Human faeces	+	–
HW961T	Human faeces	+	–
CU130P	Human faeces	+	–
CM123F	Human faeces	+	–
BV3740	Human faeces	+	–
BN499U	Human faeces	+	–
BM271M	Human faeces	+	–
XM653F	Human faeces	+	–
XX158F	Human faeces	+	–*
RC703J	Human faeces	+	–
MZ347T	Human faeces	+	–
PA268R	Human faeces	+	–
LG430I	Human faeces	+	–
HI881O	Human faeces	+	–
YU298M	Human faeces	+	–
YN438B	Human faeces	+	–
LC667G	Human faeces	+	–
YP567F	Human faeces	+	–
YO808T	Human faeces	+	–
XV898P	Human faeces	+	–*
RD177L	Human faeces	+	–
QY139W	Human faeces	+	–
IG022P	Human faeces	+	–*
ZB375B	Human faeces	+	–*
LB445E	Human faeces	+	–
ZB632B	Human faeces	+	–
SO52	Human faeces	+	–*
R923	Human faeces	+	–*
QA598G	Human faeces	+	–*
PN728G	Human faeces	+	–*
PD850L	Human faeces	+	–*
ZN244B	Human faeces	+	–*
PH844R	Human faeces	+	–
PH845S	Human faeces	+	–*
PH790A	Human faeces	+	–*
MS179P	Human faeces	+	–*
KV955A	Human faeces	+	–
ZJ638R	Human faeces	+	–*
FZ917J	Human faeces	+	–*

Table 4.2: continued...

Isolate code	Source	CadF-F2B-R1B	CadF-F2B-R1C
C. jejuni			
YJ936T	Human faeces	+	–*
YG023Q	Human faeces	+	–
YG018S	Human faeces	+	–*
MC591P	Human faeces	+	–
MC715F	Human faeces	+	–*
MD672W	Human faeces	+	–*
ZI975L	Human faeces	+	–*
MM539G	Human faeces	+	–*
LG794W	Human faeces	+	–*
DO399V	Human faeces	+	–
CJS78	Sheep	+	–
CJS79	Sheep	+	–
CJS83	Sheep	+	–*
CJS84	Sheep	+	–
CJS85	Sheep	+	–
CJS86	Sheep	+	–*
CJS87	Sheep	+	–
CJS88	Sheep	+	–*
CJS89	Sheep	+	–
CJS90	Sheep	+	–*
NZRM3242	Human faeces	+	–
KSC3516	*C. jejuni subsp. doylei*	+	–
C. coli			
M275	Human faeces	+	+
739583	Human faeces	+	+*
722841	Human faeces	+	–
736268	Human faeces	–	–
722434	Human faeces	+	–
736592	Human faeces	+	–
739584	Human faeces	+	+*
722753	Human faeces	+	+* (faint)
WA31	Water	+	+*
WA27	Water	+	+*
WA38	Water	+	–
WA36	Water	+	–
RC167B	Human faeces	+	+*
ZP028D	Human faeces	+	+*
ZH606P	Human faeces	+	+*
ZH6050	Human faeces	+	+*
ZH604N	Human faeces	+	+*
NCTC11366	*C. coli* (SS0127)	+	+*
C. lari			
AC002	Seagull	+	–*
AC081	Seagull	+	–*
AC083	Seagull	+	–*
AC087	Seagull	+	+* (faint)
AC089	Seagull	+	–*

Table 4.2: continued...

Isolate code	Source	CadF-F2B-R1B	CadF-F2B-R1C
C. lari			
NCTC11352	*C. lari* (SS0124)	+*	+*
Other *Campylobacter spp.*			
ATCC33237	*C. concisus*	–*	–*
ATCC25224	*C. curvus*	–	–
SS0120	*C. fetus*	–*	–
KSC2399	*C. fetus subsp. venerealis*	–*	–*
ATCC33236	*C. gracilis*	–	–
SS0119	*C. hyoilei*	+	+
SS0125	*C. hyointestinalis*	+	–
ATCC33238	*C. rectus*	+ (faint)	–
51146	*C. showae*	–*	–*
ATCC33562	*C. sputorum subsp. bulbulus*	–*	–
ATCC35980	*C. sputorum subsp. sputorum*	–*	–*
SS0128	*C. upsaliensis*	–	–
SS0136	*C. upsaliensis*	–	–
Non-*Campylobacter spp.*			
NZRM3205	*Aeromonas hydrophila*, ATCC7965, NCTC7812	–	–
NZRM816	*Arcanobacterium haemolyticum*, NCTC9697	–	–
SS0121	*Arcobacter butzleri*	–*	–
SS0122	*Arcobacter cryaerophilus*	–*	–
SS0116	*Bacillus cereus*	–	–
NZRM698	*Bacillus subtilus*	–	+
SS0146	*Bacteroides ureolyticus*	–	–
NZRM2565	*Branhamella catarrhalis*, ATCC25238, NCTC11020	+ (faint)	–
NZRM23	*Corynebacterium diptheriae*, ATCC19409, NCTC3984	–	–
NZRM916	*E. coli*, ATCC25922, NCTC12241	–	–
NZRM2749	*E. coli*, NCTC11560	–	–
NZRM3441	*E. coli*, O157:H7	–	–
SS0112	*Enterobacter aerogenes*	–	–
NZRM3178	*Enterococcus faecalis*	–	–
EH1087	*Erwinia herbicola*	–	–
NZRM3245	*Haemophilus influenzae*, ATCC10211	–	–
NZRM3184	*Haemophilus influenzae*, ATCC49247	–	–
NZRM3315	*Haemophilus influenzae*, ATCC49766	–	–
SS0118	*Helicobcater pylori*	–	–
SS0104	*Klebsiella pneumoniae*	–	–
SS0115	*Listeria innocua-B*	+	–*
SS0110	*Listeria ivanovii*	–	–
SS0109	*Listeria monocytogenes*	–*	–
NZRM65	*Morganella morganii*, ATCC25830, NCTC235	–	–
NZRM1036	*Neisseria gonorrhoeae*, ATCC49226	–	–
NZRM2590	*Neisseria lactamica*, ATCC23970	–	–

Table 4.2: continued...

Isolate code	Source	CadF-F2B-R1B	CadF-F2B-R1C
Non-*Campylobacter spp.*			
NZRM155	*Neisseria meningitidis*, ATCC13090	–	–
NZRM1011	*Pastuerella volantium*	–	–
NZRM805	*Plesiomonas shigelloides*	–	–
SS0103	*Proteus vulgaris*	–	–
NZRM918	*Pseudomonas aeuriginosa*, ATCC27853	–	–
SS0106	*Saccharomyces cerevisae*	–	–
SS0105	*Salmonella menston*	–	–
NZRM3226	*Salmonella typhimurium*, ATCC14028, NCTC12023	–	–
SS0114	*Shigella flexneri*	–	–
NZRM3476	*Shigella flexneri*, ATCC12022, NCTC12698	–	–
NZRM103	*Staphylococcus aureus*	–	–
NZRM2243	*Staphylococcus aureus*, ATCC29213	–	–*
NZRM87	*Staphylococcus aureus*, ATCC9144, NCTC6571	–	–*
SS0113	*Staphylococcus epidermidis*	–	–
SS0102	*Streptococcus bovis*	–	–
NZRM94	*Streptococcus dysgalactiae subsp. Equisimilis*, ATCC35666, NCTC8543	–	–
NZRM3399	*Streptococcus pneumoniae*, ATCC49619	–	–
NZRM3201	*Streptococcus pneumoniae*, ATCC6305	–	–
NZRM2723	*Streptococcus pyogenes*, ATCC19615	–	–
NZRM3250	*Streptococcus spp.*, ATCC12386	–	–
NZRM95	*Streptococcus spp.*, NCTC9603	–	–
NZRM820	*Vibrio parahaemolyticus*, ATCC43996, NCTC10884	–	–*
NZRM2603	*Yersinia enterocolitica*	–	–

4.4 Discussion

To validate the *cadF*-PCR speciation system proposed by Konkel et al. (1999a), it was necessary to select *Campylobacter* isolates from a diverse range of potential reservoirs and intermediate sources. A total of 152 environmental and clinical *C. jejuni* and *C. coli* isolates were tested. These isolates were characterised in previous studies and represent a phenotypically and genotypically diverse group (Armstrong, 1997; Calder, 1998; Yates, 1998). As the *Campylobacter* isolates chosen for *cadF* validation are geographically distinct from those tested by Konkel et al. (1999a), they provide an ideal opportunity to elucidate whether the *cadF* gene is conserved among *C. jejuni* and *C. coli* isolates.

4.4.1 DNA extraction for PCR amplification

The sensitivity of a given PCR detection system depends not only on the reaction conditions but to a great extent, on the DNA extraction method (Sheu et al., 1998). For the *cadF*-PCR pilot study, Konkel et al. (1999a) used whole cell lysates, prepared by boiling cell suspensions, as template in *cadF* gene amplifications. Although whole cell lysates were sufficient for detection of the *cadF* gene in 95% of *C. jejuni* isolates (Konkel et al., 1999a), they have previously been shown to possess a degree of unreliability resulting in false negatives (Mohran et al., 1998). Mohran et al. (1998) reported that two phenotypically distinct subgroups of *Campylobacter* strains exist with respect to their relative capacity to release intact high molecular weight DNA upon lysis in boiling water. One group, comprised of 20% of *Campylobacter* strains tested, provided mostly RNA and low molecular weight degraded DNA from whole cell lysates resulting in negative PCR results. To prevent such false negative results masking the true sensitivity and specificity of the *cadF*-PCR detection system, total genomic DNA was purified and used for *cadF* validation in the current study. A major factor affecting the inhibition of PCR with respect to DNA is the degradation of nucleic acid targets and/or primers by nucleases (Sheu et al., 1998). Two methods of DNA purification involving either guanidine thiocyanate or proteinase K-phenol/chloroform treatments were used for the *cadF* validation study. Guanidine thiocyanate has been known to inactivate endogenous

nucleases (Pitcher et al., 1989) while no detectable residual nuclease activity was reported for the proteinase K-phenol/chloroform method (Pospiech and Neumann, 1995). As a result, both methods produced DNA of a sufficient purity and yield for accurate assessment of the sensitivity of the *cadF* PCR speciation system.

4.4.2 Amplification of the *cadF* gene using the F2B and R1B primers

A total of 99.25% of *C. jejuni* isolates tested with the *cadF*-F2B-R1B primers yielded the expected 400bp PCR amplicon. This confirms the finding of Konkel et al. (1999a) that the *cadF* gene is highly conserved among a diverse group of *C. jejuni* isolates. There was no correlation between the source of *C. jejuni* and the presence of the *cadF* PCR amplicon with isolates from water, birds and sheep as well as from human faeces producing positive results. This implies that the majority of *C. jejuni* isolates possess the gene necessary for expression of the 37kDa CadF outer membrane virulence protein. As adherence to the intestinal brush border is necessary for establishing active infections (Kelle et al., 1998), these results confirm the potential for transmission of pathogenic *C. jejuni* to the human population from environmental sources.

Only one *C. jejuni* isolate (AC058) failed to produce a 400bp *cadF* PCR amplicon. This isolate was obtained from a Canada goose. The DNA obtained from this *C. jejuni* isolate was checked for purity and yield by loading into a 1% agarose gel and subjected to electrophoresis. High molecular weight DNA in the absence of smaller low molecular weight protein and RNA contamination was observed (data not shown). Furthermore, DNA from this isolate produced a profile of faint non-specific bands with the *cadF*-F2B-R1C primers (see figure 4.4, lane 5). A possible explanation for the failure to generate a *cadF* PCR amplicon with *C. jejuni* strain AC058 is that it may have acquired sequence changes as a result of the selective pressures associated with environmental stresses and as such, the *cadF* primers are prevented from specific hybridisation. Moreover, this isolate may represent a small proportion of avirulent *C. jejuni* strains that are perpetuated within the environment. Another possibility is that this isolate may have been erroneously classified and is in fact a *C. lari* strain, commonly associated with birds

(Oyarzabal et al., 1997). These results corroborate the initial findings of Konkel et al. (1999a) where 95% of *C. jejuni* were positive for *cadF*.

In total, 94.4% of *C. coli* isolates tested for the *cadF* gene with the *cadF*-F2B-R1B primers yielded a 400bp amplicon. The *cadF* gene appears to be as conserved among *C. coli* isolates as it is among *C. jejuni* isolates. As with *C. jejuni*, there was no correlation between the source of the *C. coli* isolate and the presence of the *cadF* gene. *C. coli* isolates from the clinical environment as well as from water environments routinely generated a 400bp *cadF*-F2B-R1B amplicon. One *C. coli* isolate (736268) failed to generate a *cadF*-F2B-R1B PCR amplicon. Based on the assumption that adherence to host epithelial cells is an absolute requirement for human *Campylobacter* infections, the clinical source of this isolate makes the absence of the *cadF* gene in this strain unlikely. A more feasible explanation points towards the yield of PCR detectable DNA obtained from this isolate. Attempts to PCR amplify other genes (*flaA*, 23S rRNA) using DNA from this *C. coli* isolate also failed to generate a PCR amplicon. Furthermore, no band was detected using the *C. coli*-specific *cadF*-F2B-R1C primers. This suggests that sufficient cell numbers were not used during chromosomal DNA preparation to generate high enough DNA yield to facilitate PCR detection. The endogenous nuclease inactivation properties of guanidine thiocyanate used for preparing DNA from this isolate suggest that degradation due to the presence of DNases would not have been responsible for the lack of PCR detectable DNA (Pitcher et al., 1989). Another possible explanation suggests that appropriate amounts of DNA were obtained but contained inhibitors of PCR such as substances that chelate divalent magnesium cations necessary for PCR or directly inhibit DNA polymerase (Scheu et al., 1998). Ionic detergents such as sarcosine (present in GES lysis solution) have been shown to inhibit *Taq* polymerase (Weyant et al., 1990-cited in Scheu et al., 1998). It is conceivable that washes performed on precipitated DNA from this isolate were insufficient for complete removal of sarcosine. As such, residual detergents present in purified DNA could have inhibited PCR. DNA from this isolate was not loaded into an agarose gel and electrophoresed to check for DNA purity and yield (due to time constraints). The 94.4% of *C. coli* isolates positive for the *cadF*-F2B-

R1B amplicon in this study also corroborate the result of Konkel et al. (1999a) where 83.3% of *C. coli* isolates positive for the 400bp amplicon.

Six *Campylobacter* isolates previously classified as *C. lari* were tested for the *cadF* gene. These strains encompassed five seagull isolates and the type strain. All six *C. lari* strains produced a 400bp *cadF*-F2B-R1B amplicon. Although the five seagull isolates produced relatively clean bands, the *C. lari* type strain generated a number of non-specific DNA amplicons. Due to this fact, it is not clear whether the 400bp amplicon is actually the internal fragment of the *cadF* gene or an artefact of the non-specific priming observed. Sequencing or Southern hybridisation techniques would confirm the identity of this amplicon. Alternatively, use of the rabbit anti-37Kda serum derived by Konkel et al. (1999a) for immunoblot analysis would confirm the presence or absence of the *cadF* protein from *C. lari* isolates. As *C. lari* strains were not tested by Konkel et al. (1999a) for the *cadF* gene, no previous data was available for comparison. However, *C. lari* strains have previously been implicated in a small proportion of human *Campylobacter* infections (Skirrow, 1990; Simor and Wilcox, 1987; Reed and Williams, 1998). Therefore, it is not inconceivable that some *C. lari* strains would possess genes encoding the necessary adhesins for adherence and subsequent invasion of the epithelium. In addition, the multifactorial nature of *Campylobacter* virulence suggests a number of gene products are required and adhesins other than CadF may be important for pathogenesis of *C. lari* isolates. A *cadF* detection rate of 100% in *C. lari* isolates however, is unexpectedly high given their rarity of isolation from campylobacteriosis patients. Of the five seagull isolates of *C. lari*, each had been presumptively identified as such by a negative sodium hippurate hydrolysis test and a negative *flaA* (pg50-pg3) PCR result (Calder, 1998). Sodium hippurate hydrolysis tests have been shown to be relatively unreliable for differentiating *C. jejuni* from other *Campylobacter* species (Morris et al., 1985; Totten et al., 1987). Similarly, whole cell lysates were used for *flaA* PCR by Calder (1998) and have also been shown to be unreliable as previously mentioned (Mohran et al., 1998). Therefore, it is possible that these *C. lari* isolates may in fact be *C. jejuni* isolates that have provided false negative hippurate hydrolysis and *flaA*-PCR results. Alternatively, these *C. lari* isolates may not have been sufficiently purified and

may have contained a mixture of *C. lari* and *C. jejuni* cells which may not detectable by whole cell lysates but was detected by *cadF* PCR using pure DNA in the current study. The testing of a significantly large number of confirmed *C. lari* isolates for the *cadF* amplicon is required if an accurate representation of the presence of this gene in *C. lari* is to be elucidated.

To validate the specificity of the *cadF* PCR detection system for *C. jejuni* and *C. coli* isolates, it was necessary to test a range of other *Campylobacter* species for the *cadF* 400bp *cadF*-F2B-R1B amplicon. From a total of 13 other *Campylobacter* species tested, three produced a 400bp amplicon. These were from *C. hyoilei*, *C. hyointestinalis*, and *C. rectus*. Of these three species, *C. hyointestinalis* has previously been implicated with enteric disease in humans (Skirrow, 1990). Figure 4.2 showed that the band obtained from *C. hyointestinalis* is similar in intensity to the *C. jejuni* positive control. In contrast, to this result, Konkel et al. (1999a) failed to generate an amplicon with a strain of *C. hyointestinalis*. However, the strain of *C. hyointestinalis* and the PCR conditions used between the study by Konkel et al. (1999a) and the current study were different and could have resulted in the difference observed. Immunoblot analysis of the strain used by Konkel et al. (1999a) revealed no cross-reactivity with the rabbit anti-37kDa serum suggesting the absence of the CadF protein from this *C. hyointestinalis* strain. The 400bp amplicon generated by *C. rectus* was faint and may have disappeared with a change in magnesium concentration. Fermer and Engvall (1999) found that a strain of *C. mucosalis* produced a faint band of correct size when using a set of primers specific for thermotolerant *Campylobacter spp.* This band subsequently disappeared when the $MgCl_2$ concentration was lowered. *C. hyoilei* is a relatively recently characterised species and is considered to be identical to *C. coli* (Vandamme et al., 1997). Therefore, it is not surprising that this isolate amplifies the *cadF* internal gene fragment. Six other *Campylobacter* species produced non-specific DNA fragments with the *cadF*-F2B-R1B primer set. These non-specific bands were either much larger or much smaller than the expected size amplicon and are easily distinguishable from *C. jejuni* and *C. coli* amplicons. The presence of non-specific bands from non-*C. jejuni* and *C. coli* strains were also encountered by Waage et al. (1999) in a nested PCR assay designed for specific

amplification of the intergenic region between the *flaA* and *flaB* genes from *C. jejuni* and *C. coli* isolates. Decreasing the primer concentration was found to minimise this problem (Waage et al., 1999). This was not trialed in the current study due to time constraints.

From a total of 49 non-*Campylobacter* strains tested, two produced a 400bp amplicon. These were from *Branhamella cattharalis* and *Listeria innocua-B*. The 400bp amplicon from *B. cattharalis* was very faint and unlikely to represent a true *cadF* amplicon. The amplicon produced by *L. innocua-B* was much stronger but would require confirmation as a *cadF* internal gene fragment by sequencing or Southern hybridisation. Two other *Listeria* species tested for *cadF* were negative for a 400bp amplicon. This gives rise to the possibility that the 400bp fragment observed by *L. innocua-B* is a non-specific artefact of the PCR with no potential conservation of the *cadF* gene apparent amongst *Listeria* species. Incidentally, *L. monocytogenes* produced a non-specific band with the *cadF*-F2B-R1B primer set. This amplicon was considerably larger than the expected 400bp fragment. Two *Arcobacter* species also generated large non-specific bands. These bands are easily distinguishable from the band produced by the *C. jejuni* positive control (figure 4.3). The cadF-F2B-R1B primer set is useful for determining virulence in pure cultures of *C. jejuni* and *C. coli*, but would be unreliable for specific detection from environmental samples containing other organisms such as *L. innocua-B*.

4.4.3 Amplification of the *cadF* gene using the F2B-R1C primers

Results of *cadF*-PCR detection using the *cadF*-F2B-R1C primer pair showed a greater degree of variability than those obtained with the *cadF*-F2B-R1B primers. The cadF-F2B-R1C primer set was developed by targeting a site containing a stretch of bases that appeared to be unique to the *cadF* gene in *C. coli* M275 (Konkel et al., 1999a). From a total of 18 *C. coli* isolates tested using this primer pair, 12 (67%) produced the 450bp *C. coli* specific amplicon. This percentage of positive *C. coli* isolates is considerably lower than the 83.3% positive *C. coli* isolates reported by Konkel et al. (1999a). Two major possibilities could account for this variation. Firstly, the six *C. coli* isolates that failed to produce a 450bp amplicon may have been falsely classified as *C. coli* and could in fact be *C. jejuni*. All six PCR negative *C. coli* isolates were differentiated from *C. jejuni* based

solely on sodium hippurate hydrolysis tests (Armstrong, 1997). The previously mentioned unreliability of this biochemical test (Morris et al., 1985) makes this explanation a distinct possibility. A second, possibility is that the *C. coli*-specific regions of the *cadF* gene described by Konkel et al. (1999a) is not conserved amongst all *C. coli* isolates. As only *C. coli* M275 was sequenced by Konkel et al. (1999a), the presence of identical sequences in other *C. coli* isolates could not be elucidated. There was no correlation between *C. coli cadF*-F2B-R1C amplicon negative strains and source of isolation, with both clinical and environmental *C. coli* isolates represented in the negative samples. Of the 12 *C. coli* isolates positive for a 450bp amplicon, all but the M275 control strain produced a second amplicon of approximately 650bp in size. This result was highly reproducible with modifications in the chemical and physical parameters doing little to eliminate this extra band. The intensity of this second band was identical to the expected 450bp DNA fragment (figure 4.5). This suggests that that *cadF*-R1C primer may have a second hybridisation site in some *C. coli* isolates downstream of the first binding site, resulting in the second larger amplicon. This second band was not reported for any of the *C. coli* isolates tested by Konkel et al. (1999a). It is not inconceivable that the presence of this extra amplicon is a genotype unique to these New Zealand *C. coli* isolates.

From 134 *C. jejuni* isolates tested for the *C. coli*-specific amplicon, one (0.7%) unexpectedly produced the correct size amplicon. This result suggests a relatively high level of specificity for the absence of this DNA fragment from *C. jejuni* isolates. The one clinical isolate (770928) that produced a 450bp amplicon has been well characterised and confirmed as *C. jejuni* by phenotypic and molecular tests (Jankovic, 1999). It is unclear whether or not this strain possesses the unique *C. coli cadF* gene sequence. The confirmation of this amplicon as a region of the *cadF* gene or otherwise is required. Unfortunately time constraints did not permit the verification of selected *cadF* amplicons in this thesis. Almost half (46.6%) of all *C. jejuni* isolates tested with the *cadF*-F2B-R1C primers produced non-specific banding patterns (figure 4.4). Many of these bands were in close proximity to the 450bp region and made differentiation based on amplicon length difficult. Although no bands were deemed to be of the required 450bp, should a *C. jejuni*

isolate produce a 450bp amplicon, differentiation between *C. jejuni* and *C. coli* isolates based solely on *cadF* PCR results could not be achieved. Waage et al. (1999) reported a similar problem with a *flaA* and *flaB* targeted PCR system where isolates not expected to produce an amplicon generated non-specific bands of a similar size to the expected fragment.

Of the presumptive *C. lari* isolates tested for the *C. coli*-specific amplicon, two generated a 450bp fragment. Both of these however, also generated a series of non-specific bands and as such, the identity of the 450bp amplicon as a *cadF* sequence cannot be inferred. In addition, those *C. lari* isolates that failed to produce a 450bp amplicon generated non-specific banding patterns similar to those obtained with *C. jejuni* isolates. This result, combined with the positive results from the *cadF*-F2B-R1B primer set, gives further strength to the earlier suggestion that these may in fact be *C. jejuni* isolates.

From 13 other *Campylobacter spp.* tested for the *C. coli*-specific *cadF* band, only *C. hyoilei* produced an amplicon of the appropriate size. This result further confirms the high level of identity between *C. coli* and *C. hyoilei* isolates. Three other *Campylobacter* species generated non-specific bands. None of these bands were within the 450bp region. The constant amplification of non-specific bands from non-target organisms appears to be the largest variable affecting the sensitivity of the *cadF* speciation system. Out of 49 non-*Campylobacter* isolates tested with the *cadF*-F2B-R1C primers, only *Bacillus subtilus* generated a 450bp amplicon. However, this particular strain did not produce a 400bp amplicon with the *cadF*-F2B-R1B primers. This indicates that *B. subtilus* does not possess a *cadF* gene and that the 450bp amplicon is the result of non-specific DNA amplification. Further non-specific bands were produced by two species of *S. aureus*, *V. parahaemolyticus* and *L. innocua-B.* All were much larger than 450bp and did not appear to be *cadF*-specific fragments. Of the non-*Campylobacter* species tested by Konkel et al. (1999a) that were also tested in the current study, no amplicons were generated using either primer set confirming these earlier results.

4.4.4 Specificity of *cadF*-PCR detection systems

This validation study was undertaken with three specific objectives for evaluation of the efficacy of the PCR assay based on the *cadF* virulence gene for the differentiation among and between *C. jejuni* and *C. coli* isolates. The first objective was to assess the discriminatory power of *cadF* PCR to specifically differentiate *C. jejuni* and *C. coli* from other *Campylobacter spp.* The results described in this chapter indicate that the specificity of *cadF* PCR for *C. jejuni and C. coli* isolates may not be 100% as reported by Konkel et al. (1999). Although only *C. jejuni* and *C. coli* produced the most consistent results, the potential for *C. lari*, *C. hyointestinalis* and *C. hyoilei* strains to also possess the *cadF* gene highlights the requirement for further investigation of these strains. The fact that unexpected *cadF*-PCR amplicons were not confirmed as the appropriate gene target is a major limitation of this validation study. The second objective for *cadF* validation was to demonstrate the ability of the dual primer sets F2B-R1B and F2B-R1C to distinguish *C. jejuni* and *C. coli* isolates. Although the sequences amplified using the *cadF*-F2B-R1C primer pair appear to be relatively conserved among *C. coli* isolates, the presence of non-specific banding patterns in close proximity to the *C. coli* specific amplicon region obtained from *C. jejuni* isolates highlights the need for the use of an amplicon confirmation method in conjunction with the initial PCR. This, however, decreases the rapidity with which positive results would be obtained. The final objective for *cadF* validation was to evaluate the reliability of *cadF* PCR for determining potential virulence. From a total of 108 clinical *C. jejuni* and *C. coli* isolates of proven virulence tested using the F2B-R1B primers, 99% were positive for the *cadF* gene fragment. The one isolate that was negative was likely to be due to problems with PCR inhibition rather than the absence of the *cadF* gene. These results unequivocally show that the *cadF* gene is ideally suited for determining virulence potential. The *cadF* PCR system could prove to be a useful tool for the speciation of *C. jejuni* and *C. coli* isolates in conjunction with other *Campylobacter* speciation methodologies. However, its reliability for specific detection of *C. jejuni* and *C. coli* from environmental samples where an excess of non-target nucleic acid template may be present is questionable, due to the consistent amplification of non-specific bands.

Chapter V

Characterisation of Environmental *Campylobacter* Isolates

5.1 Introduction

The efficient and accurate speciation and typing of a given set of organisms is essential for the success of epidemiological studies. Epidemiological typing is a powerful tool that enables the determination of the cause and extent of disease outbreaks, the identification of sources and transmission modes and provides insights into the relationships between isolates (Maslow and Mulligan, 1996)

5.1.1 Properties of an ideal typing system

A number of basic criteria for the evaluation of the effectiveness of an epidemiological typing system have been identified. These include typeability, reproducibility, discriminatory power, availability, cost, technical requirements and speed (Maslow and Mulligan, 1996). Typeability is described as the ability of a given typing system to produce either a positive or negative result for each isolate, void of ambiguity. A lack of typeability in a typing system would severely limit its routine use. Reproducibility refers to a method's ability to produce an identical result during repeated tests, in the same or independent laboratories. The discriminatory power of an epidemiological typing system is essential for the success of the method and refers to the ability to differentiate between epidemiologically unrelated strains.

A number of logistical criteria often govern whether a typing system is used or not. The availability of the method for routine use as well as the necessary technical requirements

such as equipment and experienced personnel with expertise in interpreting results are important considerations. Furthermore, techniques that keep costs to a minimum are favoured by most laboratories. In epidemiological studies such as those attempting to identify the source of a disease outbreak, the speed with which positive results can be obtained is vital for minimising health risks and thus will be the most preferred typing method used (Maslow and Mulligan, 1996). Although an ideal typing method would possess all of the above criteria, currently available methods have their limitations. Therefore, the choice of epidemiologic typing method must be selected with respect to the purpose for which it is to be used (Maslow and Mulligan, 1996). A vast range of speciation and typing methods have been used for *Campylobacter* species with varying levels of success.

5.1.2 *Campylobacter* speciation methods

To the generic level, *Campylobacter spp.* are presumptively identified as such by the observation of Gram negative, motile, curved rods that give a positive reaction by the oxidase test. Organising isolates of *Campylobacter* is problematic, with many of the phenotypic tests used for differentiation often returning variable results. For instance, as *Campylobacter spp.* are relatively asaccharolytic, fastidious nutritional requirements are limited in providing any degree of discrimination (Linton et al., 1997). Table 5.1 indicates the most commonly used biochemical tests for the speciation of *Campylobacter spp.*

Table 5.1: Biochemical and physiological tests for speciating organisms of the genus *Campylobacter* (adapted from On, 1996)

Organism	Hippurate hydrolysis	Nalidixic acid resistance	Catalase production	Cephalothin resistance
C. jejuni	+	S	+	R
C. coli	–	S	+	R
C. lari	–	R	+	R
C. upsaliensis	–	S	+/–*	S
C.hyointestinalis	–	S	+	S

+ = positive – = negative R = resistant S = sensitive * = A weak positive result is common among *C. upsaliensis* isolates

As can be elucidated from table 5.1, the differentiation of the three most commonly isolated and disease causing *Campylobacter spp.*, namely *C. jejuni*, *C. coli* and *C. lari*, is dependent on only a few tests. *C. lari* is separated from *C. jejuni* and *C. coli* almost exclusively by resistance to nalidixic acid (Totten et al., 1987). *C. jejuni* is typically differentiated from *C. coli* and *C. lari* by a positive sodium hippurate hydrolysis test. *C. jejuni* is the only *Campylobacter spp.* that possesses the *hip* gene (Linton et al., 1997). The *hip* gene encodes the enzyme hippuricase which facilitates the hydrolysis of sodium hippurate to benzoic acid and glycine (Morris et al., 1985). Ninhydrin reagent is used as an indicator for the presence of glycine, which if present, results in the appearance of a deep purple colour. This deep purple colour is indicative of the presence of the hippuricase enzyme and hence the identification of the organism as *C. jejuni*. *C. coli* is differentiated from *C. jejuni* by a negative sodium hippurate hydrolysis reaction and from *C. lari* by sensitivity to nalidixic acid. A major limitation of these phenotypic tests is that the interpretation of results is subjective. In addition, each test is not definitive and the false identification of isolates is a distinct possibility. A typical example is the presence of sodium hippurate hydrolysis negative *C. jejuni* isolates (Totten et al., 1987). These isolates would normally be erroneously classified as *C. coli*. In addition, the presence of nalidixic acid resistant *C. jejuni* isolates have also been reported (Reed and Williams, 1998), resulting in the false classification of *C. jejuni* as *C. lari*. The development of definitive tests that provide a high level of discrimination between *Campylobacter spp.* are essential for epidemiological analyses.

An alternative that has been used to phenotypic (biochemical and physiological) speciation techniques is the use of genotypic speciation methods. Genotypic speciation methods are reported to have a greater level of reliability than phenotypic methods, as the prime target, DNA, is common to all living organisms. The majority of genetic speciation methodologies are based upon the amplification of species-specific regions of DNA using PCR. *Campylobacter* speciation techniques using PCR are detailed in chapter IV.

5.1.3 Phenotypic typing methods

Phenotypic typing methodologies detect characteristics that are expressed by the organism. These methods are dependant on the use of identical test conditions between individual tests and between different laboratories. this is in order to avoid variation in results due to the phenotypic properties of the cell being switched on or off in response to external pressures. A number of phenotypic methods have been developed for distinguishing *Campylobacter* isolates to the species or sub-species level.

5.1.3.1 Biotyping

Biotyping of *Campylobacter* isolates is a relatively simple method with low to moderate discrimination (Patton and Wachsmuth, 1992). Typical biotyping schemes use a series of biochemical tests addressing the presence or absence of a particular function. Each positive or negative result is assigned a numerical value or a zero for positive and negative results respectively. The sum of all tests performed provides a numerical value that is referred to as the isolate biotype. Biotyping is unsuitable for detailed epidemiologic investigations and normally only separates isolates to the genus or species level (Maslow and Mulligan, 1996). Two independent biotyping schemes are commonly used. The Preston biotyping scheme employs 12 tests which includes growth at 28°C, sodium hippurate hydrolysis and ten individual antibiotic susceptibility tests (Bolton et al., 1984). Using this method, 1000 *Campylobacter* isolates were speciated as 779 strains of *C. jejuni*, 183 strains of *C. coli*, and 38 strains of *C. lari*. Among the *C. jejuni* isolates, 55 biotypes were elucidated (Patton and Wachsmuth, 1992). The Preston biotyping method results in a four digit code for each strain which collectively indicates both the species of *Campylobacter* and the specific biotype (Bolton et al., 1984). However, for distinguishing epidemiologically related strains, the Lior scheme is most often utilised (Patton and Wachsmuth, 1992). This method is based on sodium hippurate hydrolysis, rapid H_2S production and DNase activity (Patton et al., 1991). Although biotyping provides a rapid presumptive identification of commonly encountered thermotolerant *Campylobacter* strains within a short space of time (4-24h), reproducibility of certain tests is poor and highly dependant on a number of factors (Wareing et al., 1996; On and

Holmes, 1991). Among these, the age of the culture media, time of incubation and inoculum size are the most important (On and Holmes, 1991).

5.1.3.2 Auxotyping

Similar to biotyping, auxotyping makes use of the nutritional requirements of *Campylobacter spp.* for distinguishing between isolates. The development of specific, chemically defined, media has allowed for standard auxotyping of *Campylobacter spp.* (Patton and Wachsmuth, 1992). Auxotyping has the advantages of having no specialised equipment, the capabilities for testing large numbers of isolates simultaneously and consistency of results between laboratories (Patton and Wachsmuth, 1992). Its use however, is limited with regards to separating *Campylobacter* strains due to the lack of auxotrophic requirements among *Campylobacter* isolates (Yates, 1999).

5.1.3.3 Bacteriophage typing

Bacteriophage typing methods target the susceptibility of *Campylobacter* isolates to lytic phages. Species and strain specific bacteriophage bind to cell receptors on *Campylobacter* isolates and induce cell lysis, which is indicated by the formation of plaques on a bacterial lawn. The use of numerous specific bacteriophages against each isolate provides a phage pattern that is used to give phage type (Salama et al., 1990). Numerous phage typing systems have been developed, each using different numbers and types of phages. Grajewski et al. (1985) utilised 14 phages to type 94.5% of *C. jejuni* and *C. coli* isolates, which resulted in 77 unique phage patterns among 255 unrelated isolates. Phage typing has been favoured by some researchers due to the relatively inexpensive typing reagents required, which can be prepared locally without the need for specialist procedures and equipment (Bolton and Owen, 1996). This method has been described as having a moderately simple methodology with a moderate level of discrimination (Patton and Wachsmuth, 1992).

5.1.3.4 Serotyping

Serotyping is one of the oldest and most widespread of all typing systems used (Maslow and Mulligan, 1996). It detects antigenic determinants expressed on the surface of

bacterial cells using specific antisera. Two method of serotyping have been standardised by the International Committee on Serotyping *Campylobacter* (Patton and Wachsmuth, 1992). These are, firstly, the system of Penner which is based on heat-stable (HS) antigens and uses the *C. jejuni* lipopolysaccharide (LPS) as the primary HS antigen to facilitate the differentiation of isolates (Penner and Hennessey, 1980-cited in Patton and Wachsmuth, 1992). The HS system identifies 60 serotypes of which 42 are from *C. jejuni* and 18 from *C. coli* strains. HS antisera are specific for each species and allows the separate serotyping of individual species (Patton and Wachsmuth, 1992). The second method is the Lior system, based on heat-labile (HL) antigens and uses outer membrane proteins such as flagella as markers for distinguishing isolates (Lior et al., 1982-cited in Bopp et al., 1985). The HL serotyping scheme identifies 108 serotypes including eight amongst *C. lari* isolates (Patton and Wachsmuth, 1992). Serotyping has been extensively used by researchers to correlate human outbreak strains with implicated source strains (Bradbury et al., 1984; Bopp et al., 1985), as well as for studying the prevalence of specific serogroups within specific environments such as chickens and Guillain-Barré Syndrome (GBS) patients (Hood et al., 1988; Wassenaar et al., 2000). Serotyping has the major limitations of relatively low discriminatory power and non-typeability of many strains (Maslow and Mulligan, 1996). Nevertheless, serotyping is considered the gold standard by many researchers and is routinely used as a reference point for comparing other techniques (Bolton and Owen, 1996; Lind et al., 1996). Consequently, serotyping is seldom used on its own as a typing method.

5.1.3.5 Antibiotic susceptibility typing (Resistotyping)

Antimicrobial susceptibility typing or resistotyping is routinely used for testing many bacteria by microbiology laboratories (Maslow and Mulligan, 1996). The success of resistotyping is dependant on an organism's resistance to a wide range of antimicrobial agents. The antimicrobial agents suggested for use in susceptibility assays are often effective against *Campylobacter* strains resulting in a limited diversity of patterns (Patton and Wachsmuth, 1992). Hence, the choice of antimicrobial agents is integral to the discriminatory power of the typing scheme. A vast array of antibiotics have been used for testing susceptibility of *Campylobacter* strains. These include tetracycline,

erythromycin, metronidazole, clindamycin, chloramphenicol, ampicillin, kanamycin, ciprofloxacin, gentamicin and nalidixic acid (Hof et al., 1982; Freydiere et al., 1984; Akhtar, 1998; Berndtson et al., 1996; Ansary and Radu, 1992; Koenraad et al., 1995c; Aarestrup et al., 1997; Gaudreau and Gilbert, 1997; Huysmans and Turnidge, 1997; Thwaites and Frost, 1999; Smith et al., 1999; Saenz et al., 2000). In many of these studies the resistance profiles showed good correlation with the source of the *Campylobacter* isolates suggesting a common link between source of isolation and antibiotic resistance patterns. In addition, resistotyping has been shown to correlate well with the results of other typing methods (Maslow and Mulligan, 1996). A study by Ribeiro et al. (1996) where tetracycline, metronidazole, 2,3,5-tetrazolium chloride, 5-fluorouracil, sodium arsenite and nalidixic acid were used in a resistotyping scheme, found an excellent level of reproducibility with test variation of <2%. From a total of 328 *Campylobacter* isolates tested, 35 resistotypes were observed, with the largest group comprising 22% of isolates. Resistotyping was found to be convenient, simple to perform, rapid, highly discriminatory, reproducible and inexpensive, making it well suited for use by local laboratories (Ribeiro et al., 1996). It was for these reasons that this resistotyping scheme was the phenotypic typing method of choice in the current study. A major limitation of this method is that it cannot unequivocally confirm that isolates are identical although it can show that isolates are distinct (Ribeiro et al., 1996). However, when used in conjunction with one or more additional typing methods, resistotyping can be a useful means of epidemiological typing.

5.1.3.6 Other phenotypic typing methods

A small number of other phenotypic typing methods have been used to characterise *Campylobacter* strains with limited success. These include lectin typing, bacteriocin typing and protein profiling. Lectin typing uses lectins which are proteins or glycoproteins mainly derived from plants that react with specific combinations of carbohydrates. These reactions result in specific agglutination patterns that are used to distinguish *Campylobacter* isolates. This method has limited discriminatory power with a large proportion of strains often grouped to a single lectin type (Patton and Wachsmuth, 1992). Bacteriocin typing is similar to phage typing and uses the inhibition of growth of

Campylobacter isolates to specific bacteriocins to group isolates. Although a relatively simple method, bacteriocin typing shows little or no discrimination (Patton and Wachsmuth, 1992). Protein profiling separates cellular or membrane proteins according to their molecular weights using polyacrylamide gel electrophoresis (PAGE). The similarity of protein profiles indicates relatedness of isolates. Protein profiling requires specialised reagents and equipment, results are often complex and difficult to interpret and discrimination is only low to moderate for *Campylobacter* isolates (Patton and Wachsmuth, 1992; Maslow and Mulligan, 1996)

5.1.4 Genotypic (Molecular) typing methods

Genetic typing methodologies examine an organism's DNA directly. These methods can discriminate between isolates regardless of whether the gene or genes in question are expressed or not. A number of molecular typing techniques have been applied to *Campylobacter* isolates.

5.1.4.1 Restriction endonuclease analysis (REA)

REA is a technique in which purified chromosomal DNA is digested with a restriction endonuclease, subjected to electrophoresis through an agarose gel, stained in ethidium bromide (EtBr) and the resultant pattern visualised with UV light. The choice of restriction endonuclease is integral to the success of this method. Lind et al., (1996) trialed seven different endonucleases for REA of *C. jejuni* isolates from outbreaks. Of these, only one (*Hae* III) generated a DNA profile of acceptable resolution. Patton et al. (1991) were able to sort 22 *Campylobacter* isolates into nine distinct profiles using the enzymes *Bgl* II and *Xho* I. Bradbury et al. (1984) found REA sufficiently sensitive to confirm a suspected cattle transmitted campylobacteriosis outbreak. In this study, restriction profiles of cattle and human isolates were indistinguishable. The major difficulty encountered with REA is that chromosomal restriction patterns can consist of hundreds of bands making interpretation of results difficult and time consuming (Maslow and Mulligan, 1996). In addition, the presence or absence of plasmids can result in isolates with genetically identical chromosomal DNA producing different REA profiles.

5.1.4.2 Ribotyping

Ribotyping measures chromosomal differences by hybridisation of an rRNA-DNA probe to restriction enzyme digested genomic DNA. The ribosomal genes are used as a probe because they are highly conserved among bacteria and there are generally multiple targets per genome (Maslow and Mulligan, 1996). Fitzgerald et al. (1996) used ribotyping of the 16S rRNA gene for successful differentiation of 261 isolates belonging to all HS serotypes of *C. jejuni*, into 77 ribotypes. Patton et al. (1991) reported the differentiation of 22 *Campylobacter* isolates into nine specific ribotypes. Typically, three to eight bands ranging from 2-24kb are observed for ribotypes of *Campylobacter* strains (Fitzgerald et al., 1996; Fayos et al., 1992). This method has a discriminatory power that is directly proportional to the number of ribosomal loci present and the genetic diversity of the organism (Maslow and Mulligan, 1996). Although ribotyping is useful for characterisation of most bacterial pathogens, the procedure is complex and time consuming. This has limited its routine use for typing of *Campylobacter* isolates (Patton et al., 1991).

5.1.4.3 Multi-locus enzyme electrophoresis (MLEE or MEE)

MLEE or MEE measures the electrophoretic migration distance of selected enzymes. The variation in migration distances results in a profile referred to as the electrophoretic type (ET) (Maslow and Mulligan, 1996). The differences in electrophoretic mobility are thought to relate directly to allelic variation in the structural gene locus for each enzyme (Patton and Wachsmuth, 1992). Aeschbacher and Piffaretti (1989) analysed 125 *C. jejuni* and *C. coli* isolates from human and animal hosts using MLEE. A total of 64 electrophoretic types representing two distinct clusters (one for *C. jejuni* isolates and one for *C. coli* isolates) were identified, demonstrating a high level of discrimination. Although highly sensitive, MLEE is complex, dependant on the number of enzymes analysed and is time consuming (Patton and Wachsmuth, 1992). Other typing methods that have an equally high discriminatory power but are less technically complex are often preferred to MLEE for typing bacterial strains (Maslow and Mulligan, 1996).

5.1.4.4 Plasmid profiling

Plasmid profiling involves the extraction and separation of plasmid DNA from chromosomal DNA, digestion of the plasmid DNA by restriction endonucleases and separation of DNA fragments by agarose gel electrophoresis. This method of typing is only applicable to strains that contain plasmid DNA. Approximately 19-53% of *C. jejuni* and *C. coli* isolates have been identified as containing plasmids (Patton and Wachsmuth, 1992). Plasmid profiling of *Campylobacter spp.* has given variable results with some studies reporting a high level of discrimination between *C. jejuni* isolates containing plasmids of the same size (Bopp et al., 1985), while others have suggested that it is only infrequently useful due to a lack of plasmid DNA from important outbreak strains (Patton et al., 1991). Although plasmid profiling has no quantitative means for comparing results between laboratories, it is technically simple, inexpensive and rapid (Patton and Wachsmuth, 1992; Maslow and Mulligan, 1996).

5.1.4.5 PCR-RFLP

PCR-restriction fragment length polymorphism (RFLP) analysis is a useful typing method whereby a PCR amplicon is digested with a restriction endonuclease. The digested PCR amplicon is subjected to electrophoresis, the gel stained in Etbr and restriction fragments analysed by visualisation under UV light. PCR-RFLP based typing systems are simple to use and provide a rapid method of bacterial typing. For characterisation of *Campylobacter* strains, two major gene targets have been used for RFLP analysis.

PCR-RFLP typing of *Campylobacter* isolates using DNA sequences encoding the 16S and 23S rRNA genes has been successfully used for speciation and discrimination of *Campylobacter* isolates. Hurtado and Owen (1997) were able to rapidly identify 118 isolates representing 15 species of *Campylobacter* and four species of *Arcobacter* by the PCR amplification of an internal region of the 23S rRNA gene and subsequent restriction digestion with three endonucleases, *Hpa* II, *Cfo* I, and *HinF* I. Individual profiles produced by the three restriction endonucleases were capable of separating *Arcobacter spp.* from *Campylobacter spp.*, differentiating between *C. coli, C. lari, C. upsaliensis, C.*

fetus, C. jejuni, C. hyointestinalis and *C. helveticus* as well as producing strain-specific profiles amongst *C. jejuni* and *C. coli* isolates. Similarly, PCR-RFLP analysis by amplification of a 283bp amplicon of 16S rRNA gene and restriction digestion with a range of six different endonucleases allowed the discrimination of *Campylobacter spp.* from closely related genera as well as discrimination between *Campylobacter* strains (Cardarelli-Leite et al., 1996).

The *flaA* gene has been extensively used for RFLP analysis of *Campylobacter* strains. The high level of sequence heterogeneity within the *flaA* gene due to the V1 variable region makes it an ideal target for strain discrimination (Fischer and Nachamkin, 1991). The variable region of the *flaA* gene has the potential to undergo frequent variation due to antigenic pressures (Alm et al., 1993). Nachamkin et al. (1993) developed a flagellin gene typing system where a 1.7kb *flaA* PCR amplicon was analysed for polymorphisms by digestion with the endonuclease *Dde* I. From 43 *C. jejuni* strains belonging to six common HL serotypes, 18 different RFLP patterns were observed. Similarly, Nachamkin et al. (1996) extended the above typing system by analysing 404 *Campylobacter* isolates, identifying 83 distinct flagellin types. Flagellin gene typing has subsequently been used successfully for the analysis of the diversity of *Campylobacter* strains within chicken farms as well as for assessment of the initial sources involved with broiler chicken colonisation (Chuma et al., 1997; Stern et al., 1997). Koenraad et al. (1995) used flagellin gene typing to correlate the contamination of wastewater by poultry sources. In this study, *Campylobacter* strains with RFLP patterns found in poultry were also detected in wastewater that was presumed to be contaminated solely from human and domestic sources. In addition to *Dde* I restriction profiles, a number of other endonucleases have been successfully used for flagellin gene typing. Owen et al. (1994) found *HinF* I *flaA* typing to produce reproducible, high quality separation of isolates. The endonucleases *Bgl* II and *Alu* I have also been used with some success (Linton et al., 1996; Mahendru et al., 1997). Simultaneous double digestion of *flaA* products with *EcoR* I and *Pst* I has also been used as an excellent molecular subtyping method for discrimination between human, animal and poultry isolates (Mohran et al., 1996; Owen and Leeton, 1999). The use of the *flaA* gene for sub-typing *Campylobacter* isolates is a rapid, reproducible

technique which generates sufficient discrimination to be useful as a practical typing method for clinical and epidemiologic investigations (Nachamkin et al., 1993).

5.1.4.6 Pulsed-field gel electrophoresis (PFGE)

Pulsed-field gel electrophoresis was developed in 1984 by Schwartz and Canter as a variation to agarose gel electrophoresis for the separation of large DNA fragments (Maslow and Mulligan, 1996). PFGE involves embedding organisms in agarose, lysing the organisms *in situ* and digesting the chromosomal DNA with 'rare cutting' restriction endonucleases. Slices of agarose containing chromosomal DNA fragments are loaded into the wells of an agarose gel and the restriction fragments resolved into a pattern of discrete bands within an apparatus which periodically changes (pulses) the direction of the current according to a pre-determined pattern (Tenover et al., 1995). The apparatus with which restriction fragments are resolved often varies with three PFGE methods most commonly used. These are the separation of DNA fragments by field inversion gel electrophoresis (FIGE), orthogonal field-alternating gel electrophoresis (OFAGE) or contour-clamped homogenous electric field (CHEF) (Bustamante et al., 1993). All three methods produce similar results with the CHEF system normally producing the most uniform restriction profile.

PFGE is highly discriminatory and is favoured by many researchers as the molecular typing tool of choice for epidemiologic investigations. PFGE has been successfully used for sizing of *Campylobacter* genomes (Chang and Taylor, 1990), assessing the genomic instability of *Campylobacter* isolates passaged through chickens (Wassenaar et al., 1998; Hanninen et al., 1999), identifying the level of genotypic heterogeneity among individual *Campylobacter spp.* (Bourke et al., 1996; On, 1998) and the epidemiologic typing of outbreak strains with various environmental source strains (Hageltorn and Berndtson, 1996; Steele et al., 1998; On et al., 1998; Hanninen et al., 1998a; Hanninen et al., 1998b; Hudson et al., 1999).

For PFGE to be effective, the choice of restriction endonuclease is of considerable importance. Endonucleases that cut too frequently result in a profile of hundreds of

bands which are extremely difficult to interpret. The enzyme *Sma* I is most commonly used for PFGE typing of *Campylobacter* isolates, which typically generates a profile of approximately nine bands (Ayling et al., 1996; Geilhausen et al., 1996; Gibson et al., 1996; Shi et al., 1996; Wassenaar et al., 1998). Other enzymes that have also been shown to be useful include *Sac* II (Hanninen et al, 1999), *Xho* I (Bourke et al., 1996), *Kpn* I, *Sal* I and *BamH* I (On et al., 1998; On, 1998). The major limitations of PFGE are the requirement for expensive equipment and reagents and long DNA preparation and electrophoresis times, often taking several days before results can be obtained. However, rapid PFGE protocols have been described (Gautom, 1997) and coupled with its high reproducibility, high discrimination and universal application, it is an ideal epidemiological typing method.

5.1.5 Virulence determinants

When analysing *Campylobacter* isolates from environmental sources, it is important to establish whether or not they are capable of causing disease in humans. As human volunteer studies are not always practical, the detection of virulence determinants is a useful way to assess the potential for an isolate to be pathogenic. The most simple and rapid method for detection of virulence factors is the PCR amplification of regions of DNA encoding specific virulence genes. A number of PCR-amplifiable virulence factor genes can be assessed.

5.1.5.1 The flaA gene

The *flaA* gene encoding the major protein subunit of flagella (Nuijten et al., 1992) is thought to play an integral role in the colonisation of the intestinal mucosa. PCR primers targeting an internal region of the *flaA* gene are specific for the detection of *C. jejuni* and *C. coli* which are most often implicated in campylobacteriosis cases. Hence the detection of the *flaA* gene in environmental isolates would be indicative of virulence potential.

5.1.5.2 The cadF gene

The *cadF* gene encodes an adhesin protein that is considered important for the adherence to epithelial cells for initiating pathogenesis. The *cadF* gene is outlined more fully in chapter IV.

5.1.5.3 The ciaB gene

The *ciaB* gene (*Campylobacter* invasion/internalisation antigen B) was initially described by Konkel et al. (1999b). Functional analysis of the *ciaB* gene product demonstrated its importance in the internalisation of cultured cells. The protein produced by *ciaB* was found to consist of 610 amino acids with a calculated molecular mass of approximately 73kDa. *In vitro* binding and internalisation assays revealed that a significant reduction in internalisation occurred with *ciaB* null mutants while binding was indistinguishable from the wild type strains. CiaB was found in the cytoplasm of host cells with the wild type strain while a loss of secreted proteins was observed with the mutant strain. It was concluded that the *ciaB* gene product plays a role in the secretion process required for efficient entry and internalisation of bacteria and hence pathogenesis (Konkel et al., 1999b). The detection of the *ciaB* gene from environmental isolates would indicate the potential for causing invasive disease.

5.1.6 Tissue culture assays

Tissue culture assays are an ideal way to confirm the virulence of an environmental *Campylobacter* isolate. Tissue culture assays involve the co-cultivation of host cells with the bacterial isolate to be tested for virulence. A number of cell lines are available for tissue culture experiments. The most commonly used cell lines for *Campylobacter* virulence assays are HeLa, Caco-2 and INT-407 (Goossens et al., 1992; Konkel et al., 1992; Yao et al., 1994; Harvey et al., 1999). Tissue culture experiments have been used for the elucidation of a range of *Campylobacter* pathogenic mechanisms. These include invasion (Harvey et al., 1999; Goossens et al., 1992), translocation across cell monolayers (Konkel et al., 1992), motility (Yao et al., 1994; Szymanski et al., 1995), adherence (Sylvester et al., 1996) and toxin production (Misawa et al., 1994; Misawa et al., 1995). Although useful for determining virulence and pathogenic mechanisms, tissue culture

requires specialist equipment and facilities as well as technical expertise, making it relatively inaccessible to many laboratories.

5.1.7 Objectives for characterisation of environmental isolates

The intent of research described in this section is to confirm the identity of environmental isolates (as *C. jejuni, C. coli* or *C. lari*) and assess the genetic relationships between each of these isolates. The aims are to:

1. Identify *Campylobacter* isolates to the species level using established morphological and biochemical techniques

2. Group isolates into discrete categories using a combination of antimicrobial susceptibility typing (resistotyping), *fla*A PCR-RFLP typing and pulsed field gel electrophoresis (PFGE)

3. Assess relatedness of isolates from differing environmental sources and determine possible links between these sources; PFGE, in combination with PCR-RFLP, will be used to determine the genetic relatedness between isolates of the same species

4. Evaluate the potential virulence of isolates by detection of the specific virulence factor genes *cadF, ciaB* and *flaA* using PCR

5. Further confirm virulence of environmental isolates by performing tissue culture experiments whereby environmental isolates are used to infect epithelial cell lines (as time permits).

5.2 Materials and Methods

5.2.1 Campylobacter speciation

Moist, flat, grey spreading bacterial colonies isolated through environmental sampling were streaked onto CCDA and cultured for 24-48h in preparation for morphological, biochemical and genetic identification.

5.2.2 Gram stain and motility

All environmental isolates were visualised by Gram staining in order to assess purity and ensure cells were Gram negative. Pure cultures were tested for motility using the hanging drop test.

5.2.3 Hippurate hydrolysis

An 1.0% solution of sodium hippurate was prepared in dH_2O and sterilised by autoclaving. Aliquots of 400µl were aseptically dispensed into sterile Eppendorf tubes and stored at -20°C until required.

Using a sterile wire loop, a loopful of cell culture was scraped from the surface of a CCDA plate and suspended in a tube of sodium hippurate solution. Tubes were incubated at 37°C in ambient oxygen atmosphere for 2h. Following incubation, 200µl of ninhydrin reagent (appendix II) was added and the tube incubated for a further 10min. The appearance of a deep purple colour was recorded as a positive result. Tubes showing no colour or a very faint purple colour were considered to be negative for hippurate hydrolysis (Morris et al., 1985). *C. jejuni* F38011 and *C. coli* M275 were used as positive and negative controls respectively for all hippurate tests.

5.2.4 Naladixic acid susceptibility

Naladixic acid susceptibility tests were carried out to further differentiate *C. jejuni, C. coli* and *C. lari* isolates. Gram negative, motile, hippurate hydrolysis positive and naladixic acid sensitive isolates were considered to be *C. jejuni*. Gram negative, motile,

hippurate hydrolysis negative, naladixic acid sensitive isolates were classified as *C. coli*, whereas Gram negative, motile, hippurate hydrolysis negative and naladixic acid resistant isolates were presumptively classified as *C. lari*.

5.2.5 Antimicrobial susceptibility typing (Resistotyping)

Resistotyping was performed essentially as described by Ribeiro et al (1996). *Campylobacter* isolates were streaked onto CCDA and cultured for 24-48h. Following incubation, cellular growth was collected using a sterile cotton swab (Biolab Scientific) and suspended in an Eppendorf tube containing 500µl PBS. Cell suspensions were swabbed onto Mueller Hinton agar (Remel Lenexa) (appendix I) containing 5% defibrinated sheep's blood (GibcoBRL) (MHB). A bacterial lawn was ensured by thoroughly swabbing each plate in three different directions. Plates were allowed to dry for 15min in a laminar flow cabinet prior to addition of antibiotic disks

5.2.5.1 Disk Preparation and Placement

Antibiotic disks were prepared by adding 25µl of antibiotic solution at the appropriate concentration (Table 5.2) to sterile antibiotic test assay disks (Schleicher & Scheull) (6mm diameter). Disks were frozen at -80°C overnight and subsequently freeze dried. Disks were stored at 4°C until required.

Table 5.2 Antimicrobial agents and concentrations used for resistotyping

Antimicrobial Agent	Abbreviation	Concentration Per Disk
Tetracycline hydrochloride	Tet	3µg
Naladixic acid	Nal	30µg
Metronidazole	Met	5µg
2,3,5-Tetrazolium chloride	TTC	600µg
5-Fluorouracil	5-Fl	60µg
Sodium arsenate	NaAr	20µg

Antibiotic disks were placed onto pre-inoculated MHB plates using a sterile scalpel to transfer disks from storage container to bacterial lawns. Only three disks were applied to

each plate to facilitate reading of the zones of inhibition from susceptible isolates. MHB plates were incubated for 24-48 hours at 37°C in a microaerophilic atmosphere.

5.2.5.2 Analysis of Plates

Zones of inhibition of >10mm were recorded as sensitive as suggested by Ribeiro et al (1996). Results were analysed to form a two-digit numerical resistotype code. Resistance to Tet, Nal, and Met was scored as 10, 20, and 40 respectively, whereas resistance to TTC, 5'Fl and NaAr was scored as 1, 2 and 4 respectively. The sum of all the scores resulted in the resistotype code.

5.2.5.3 Resistotype controls

C. jejuni isolates KLC4297 and KLC4300 were used as controls to ensure disks were functioning correctly. Each *C. jejuni* isolate represents the upper and lower extreme of resistotype code, pre-determined as 00 for KLC4297 and 67 for KLC4300.

5.2.6 *flaA*-RFLP typing

DNA was extracted from isolates using either one of the two methods described in section 4.2.2. Purified DNA was used as template for the amplification of the *flaA* gene by PCR.

5.2.6.1 flaA PCR

PCR reactions were prepared essentially as described in section 4.2.4 using the primers pg50 and nr2 (GibcoBRL) at a concentration of 5pmol per reaction. Amplification of the *flaA* gene consisted of 37 cycles, the first cycle was at 94°C for 1min to denature all double stranded DNA. Subsequent cycles (2-36) had three steps: 94°C for 15 sec, 55°C for 45 sec and 72°C for 1min per cycle. The final cycle (37) consisted of steps 1 and 2 from cycles 2-36, with a lengthened elongation step (72°C for 5min) to ensure all initiated amplifications were completed. A final soak step at 4°C was included to refrigerate samples until further processing. *C. jejuni* F38011 was used as a positive control for all *flaA* amplifications. Tubes containing all PCR reagents except DNA

template were used as negative controls. A 15µl aliquot of PCR product was loaded into an 1.0% agarose gel subjected to an electrical current of 100V for 1h, stained in EtBr and visualised under UV light (see section 4.2.5) in order to detect the presence or absence of the appropriate 1.7kb *flaA* amplicon.

5.2.6.2 Restriction endonuclease digestion of flaA amplicons

PCR reactions positive for a 1.7kb amplicon were digested with the restriction enzyme *Dde* I. Digestions were performed in sterile Eppendorf tubes and consisted of 10µl of *flaA* amplicon, 5 units (0.5µl) of *Dde* I (Roche) with 1.5µl of incubation buffer H (Roche) and 3µl sterile ddH_2O per reaction. Tubes were incubated at 37°C for 2-3h.

5.2.6.3 Electrophoresis of Restriction Digests

A 4% agarose gel (Seakem LE/Nusieve 3:1) was prepared as described in section 4.2.5. The entire restriction digest was mixed with 2µl 6x bromophenol blue gel dye and loaded into the pre-cast well of an agarose gel. Gels were subjected to electrophoresis at 50 volts for approximately 2h, stained in ethidium bromide and visualised under UV light. An 100bp molecular weight marker (Bio-Rad) was used as a molecular weight reference for determining fragment sizes.

5.2.6.4 Restriction Fragment Size Analysis

This was done by visually comparing RFLP profiles to each other. Restriction fragment sizes were estimated to fit into 25bp intervals using a molecular weight marker as a reference, to generate a binary code data table using Microsoft Excel. For each 25bp interval, a fragment was deemed to be present or absent and coded with a 1 or 0 respectively. Similar appearing isolates were resolved next to each other to confirm clonality of profiles. The binary table was imported into the statistical programme S-Plus and cluster analyses carried out using the unweighted pair group algorithm averages (UPGMA). This data was used to create a dendrogram to show genetic relatedness of isolates.

5.2.7 Pulsed Field Gel Electrophoresis (PFGE)

5.2.7.1 Buffers and solutions

All buffers and solutions used for pulsed field gel electrophoresis are listed in appendix III.

5.2.7.2 Preparation of Agarose Embedded Chromosomal DNA

Growth from a 24-48h CCDA plate was collected using a sterile swab (Biolab Scientific) and suspended in a test tube containing 2ml of PETT IV buffer to give a turbidity equivalent of a M^{c}Farland standard #1. The suspension was transferred to a sterile Eppendorf tube and centrifuged for 10min, 4°C at 6,600 xg. Supernatant was discarded and the pellet resuspended in 125µl of PETT IV buffer. An 1.6% solution of pulsed field certified agarose (Bio-Rad) and dH_2O was heated until dissolved and held in a 50°C water bath. Plugs were formed by pipetting a 50:50 mixture of agarose and cell suspension into a plug mould (Bio-Rad). Plugs were allowed to solidify (10-15min), and placed into sterile universal bottles containing 2ml EC lysis buffer with lysozyme (final concentration 1 mg/ml) and heat-treated Rnase (final concentration 20 µg/ml). Lysis was performed by incubation overnight in a 37°C shaking water bath. The lysis buffer was subsequently removed and the plugs immersed in 1-2 ml of ESP buffer containing proteinase K (final concentration 0.5 mg/ml) and incubated overnight at 55°C. Following incubation, plugs were transferred to sterile plastic 50 ml tubes (Corning) and washed in sterile dH_2O for 30min. A further 2-4 washes were performed in 1x TE buffer at 4°C overnight. Plugs were subsequently cut into 5-7 even slices and stored at 4°C in 1 ml of 1x TE buffer until required.

5.2.7.3 Restriction Endonuclease Digestion of DNA Plugs

Plug slices were digested using the enzyme *Sma* I (Roche). Digestions were performed in sterile Eppendorf tubes and consisted of 20 units of *Sma I* with 15µl of incubation buffer A (Roche) and 133µl of sterile ddH_2O per reaction. Tubes were incubated at 25°C overnight.

5.2.7.4 Electrophoresis of Plug Digestions by Pulsed Field

An 1.0% agarose gel was constructed by heating the appropriate amount of pulsed field certified agarose in 0.5x TBE buffer until dissolved. The gel was cast in pulsed field gel moulds and allowed to set. Digested agarose plugs were placed into the pre-cast wells of the gel and sealed with molten agarose which, when set, prevents plugs from floating out of the gel. Lambda ladder Pfg marker (New England Biolabs) was used as a weight reference in order to assess band sizes. Electrophoresis was performed on the CHEF-mapper (Bio-Rad) system with 2L of 0.5 × TBE running buffer. Electrophoretic conditions were as follows: a running temperature of 14°C, gradient of 6.0 v/cm; a run time of 22h; included angle of 120°; initial switch time of 3sec; final switch time of 30sec and a linear ramping factor.

After electrophoresis, gels were stained in dH_2O containing 20µl of EtBr (10mg/ml) for 20min with gentle shaking, and destained in dH_2O overnight. Gels were visualised using an 'Ultra Lum Electronic' UV transilluminator (254nm) and photographed with a Kodak DC120 electrophoresis documentation and analysis digital camera. PFGE profiles were analysed as described in section 5.2.6.4.

5.2.8 PCR detection of *cadF* and *ciaB* virulence genes

PCR of the *cadF* gene on environmental *Campylobacter* was performed using the F2B-R1B and F2B-R1C primer sets as described in section 4.2.4. The *ciaB* internalisation gene was PCR amplified using primers *ciaB*-F and *ciaB*-R (Table 4.2). The PCR reaction mix was as described for *cadF*-PCR (see section 4.2.4). Amplification of *ciaB* consisted of 32 cycles with denaturation for 1min at 94°C, annealing for 1min at 55°C and elongation for 5min at 72°C per cycle. PCR products were resolved in 1.5% agarose gels by electrophoresis at 100V for 1h. Gels were stained in dH_2O containing EtBr with gentle shaking for 15-20min and visualised under UV light (see section 4.2.5).

5.3 Results

5.3.1 Identification of environmental *Campylobacter* isolates

Of the 22 *Campylobacter* isolates obtained from environmental sampling, all were Gram negative and motile. The speciation of these isolates using sodium hippurate hydrolysis and naladixic acid susceptibility gave a number of conflicting results. In an attempt to clarify this, each isolate was tested using the *cadF* PCR speciation system assessed in chapter IV. The results of *Campylobacter* speciation tests and the presumptive identification of each isolate is shown in Table 5.3.

Table 5.3: Environmental *Campylobacter* isolate presumptive identifications

Isolate	Sodium hippurate hydrolysis	Naladixic acid susceptibility	*CadF*-F2B-R1B amplicon	*cadF*-F2B-R1C amplicon	Presumptive identification
AW9901	+++	S	+	–	*C. jejuni*
HW9902	+++	R	+	–	*C. jejuni*?
SW9903	+++	S	+	–	*C. jejuni*
AW9904	+++	S	+	–	*C. jejuni*
AW9905	+++	S	+	–	*C. jejuni*
HW0006	–	S	+	–	*C. jejuni*?
AW0007	–	S	+	–	*C. jejuni*?
AW0008	++	R	+	–	*C. jejuni*?
EC0009	–	R	–	–	*C. lari*
HW0010	+++	S	+	–	*C. jejuni*
HW0011	+++	S	+	+*	*C. jejuni*?
HW0012	+++	R	+	+*	*C. jejuni*?
GW0013	–	S	+	+	*C. coli*
GW0014	+++	S	+	–	*C. jejuni*
AM0015	+++	S	+	–	*C. jejuni*
AM0016	+++	S	+	–	*C. jejuni*

Table 5.3: continued...

Isolate	Sodium hippurate hydrolysis	Naladixic acid susceptibility	*CadF*-F2B-R1B amplicon	*cadF*-F2B-R1C amplicon	Presumptive identification
AM0017	+++	S	+	–	*C. jejuni*
AM0018	+++	S	+	–	*C. jejuni*
AM0019	+++	S	+	–	*C. jejuni*
AM0020	+++	S	+	–	*C. jejuni*
AM0021	+++	S	+	–	*C. jejuni*
HM0022	+++	S	+	–	*C. jejuni*

+++ = strong positive ++ = positive S = susceptible R = Resistant + = expected amplicon present – = no amplicon +* = non-specific bands present

As can be seen from table 5.3, 14 of 22 isolates were shown to be *C. jejuni* with a high degree of confidence. One isolate (GW0013) can also confidently be presumed to be *C. coli*, while one isolate (EC0009) is a *C. lari* strain. Of the remaining six isolates, uncertainty remains as to their specific identity.

5.3.2 Resistotyping results

Resistotyping was simple to perform and zones of inhibition were usually large and readily determined. When duplicate tests were performed simultaneously, the results were highly reproducible with no discrepancies observed. All 22 environmental isolates were typeable. The zones of inhibition observed for each isolate to the antibiotics used are shown in appendix IV, Table A4.1. A total of nine resistotypes were observed from a possible 64 profiles. These are indicated in Table 5.4.

Table 5.4: Number of isolates and frequency (%) for each resistotype profile identified

Resistotype	No. of isolates	Frequency
04	4	18.2%
05	2	9.1%
06	7	32.0%
07	3	13.6%
26	1	4.5%
47	2	9.1%
66	1	4.5%
67	1	4.5%
74	1	4.5%

The majority of isolates (64%) were within three resistotypes (04, 06, 07) with the largest group, resistotype 06 comprising 32% of isolates. Isolates obtained from the Avon river were split amongst six resistotypes; isolates from water samples were resistotypes 05, 06 and 26 and mussel bioaccumulation isolates resistotypes 04, 07 and 47. Isolates from the Heathcote River were diverse and split amongst five resistotypes (04, 05, 06, 67, 74). The seawater isolate belonged to resistotype 06 consistent with other water isolates. The cockle isolate produced a unique resistotype. Both isolates from the Groynes were grouped into different resistotypes. Table 5.5 indicates the resistotype and source of each isolate.

Table 5.5: Environmental *Campylobacter* isolates grouped by resistotypes and source

Isolate	Resistotype	Source
HW0011	04	Heathcote river water
GW0014	04	Groynes water
AM0016	04	Avon river mussel
AM0017	04	Avon river mussel
AW9904	05	Avon river water
HM0022	05	Heathcote river mussel
AW9901	06	Avon river water
SW9903	06	Sumner seawater
AW9905	06	Avon river water
HW0006	06	Avon river water
AW0007	06	Avon river water
HW0010	06	Heathcote river water
GW0013	06	Groynes water
AM0019	07	Avon river mussel
AM0020	07	Avon river mussel
AM0021	07	Avon river mussel
AW0008	26	Avon river water
AM0015	47	Avon river mussel
AM0018	47	Avon river mussel
EC0009	66	Estuary cockle
HW0012	67	Heathcote river water
HW0002	74	Heathcote river water

Resistance to each antibiotic was highly variable. The most notable of which was a 100% resistance to sodium arsenite. Table 5.6 shows the resistances to each antibiotic.

Table 5.6: Resistance of isolates to each antibiotic used

Antibiotic	No. of resistant isolates	% of resistant isolates
Tetracycline hydrochloride	1	4.5%
Metronidazole	5	22.5%
Naladixic acid	4	18%
2, 3, 5-Tetrazolium chloride	8	36.3%
5-Fluorouracil	15	68%
Sodium arsenite	22	100%

Using the data for resistance or susceptibility to each antibiotic, a binary data set was produced which allowed the plotting of a dendrogram showing the level of similarity between strains represented in each resistotype (Appendix IV; Figure A4.1). Isolates from resistotypes 47 and 67 were shown to have the greatest level of similarity at a similarity coefficient of 0.8 (a similarity coefficient of 1.0 indicates 100% relatedness). The three largest resistotype groups (04, 06 and 07) are represented by three distinct clusters. When compared with data from *flaA* RFLP and PFGE, the resistotype dendrogram was found to show a high degree of similarity between distantly related isolates and thus was not a good measure of isolate relatedness. Resistotyping results should not be used as a measure of isolate relatedness in future studies.

5.3.3 *flaA* PCR-RFLP typing results

Of the 22 environmental isolates analysed by PCR-RFLP, 21 (95.5%) produced the expected 1.7kb amplicon with the primers pg50 and nr2. The one isolate that did not generate an amplicon was the *C. lari* isolate (EC0009). Failure to produce a *flaA* amplicon further confirms that this isolate is not a *C. jejuni* or *C. coli* isolate. Of the 21 isolates that produced a 1.7kb amplicon, all (100%) produced RFLP profiles when digested with *Dde I.* Analysis of these RFLP profiles allowed the differentiation of 17 distinct restriction patterns. Typical *flaA* PCR amplicons and a representative sample of *Dde I* restriction profiles are shown in figure 5.1.

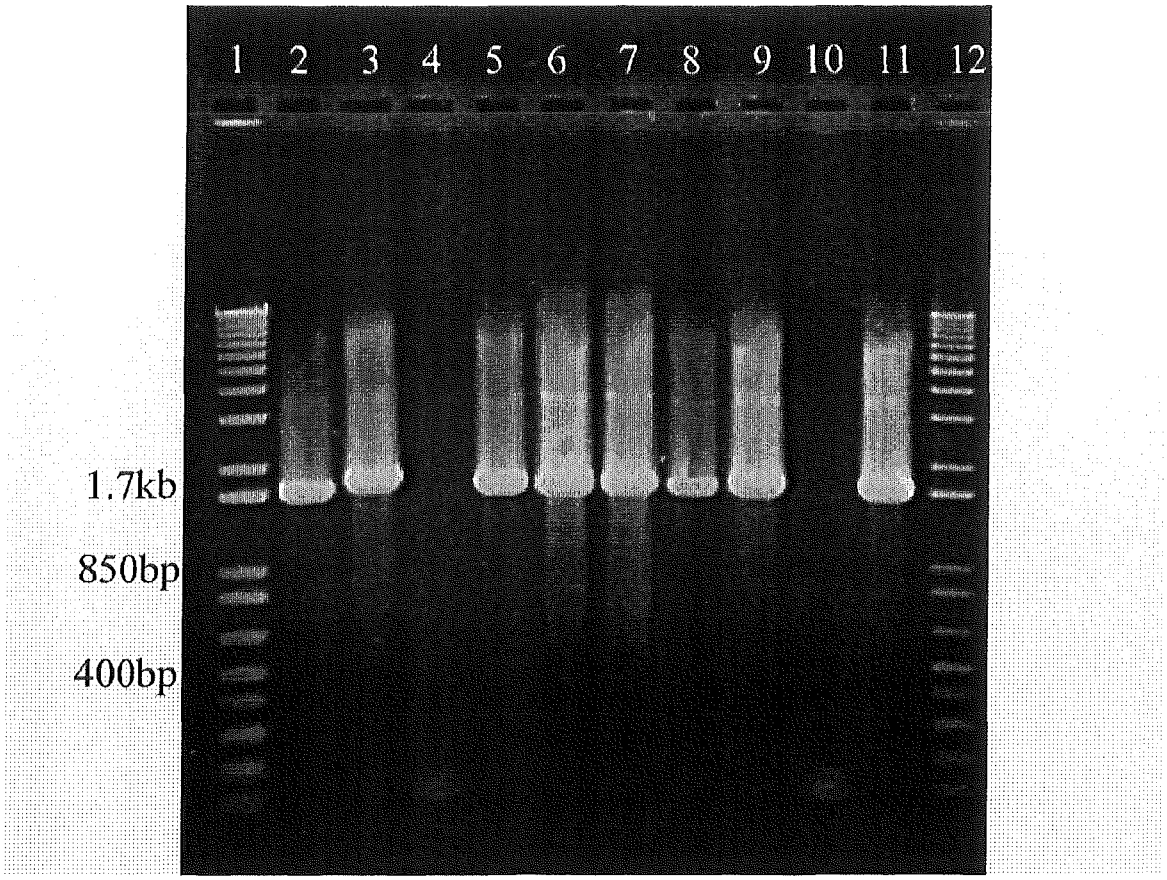

Figure 5.1a: Agarose gel of PCR amplicons generated using the *flaA* specific primers pg50 and nr2 with DNA from environmental *Campylobacter* isolates. Lane 1, 1kb plus marker (GibcoBRL); lane 2, HW9902; lane 3, HW0006; lane 4, EC0009; lane 5, HW0010; lane 6, HW0011; lane 7, HW0012; lane 8, GW0013; lane 9, GW0014; lane 10, Negative control (No DNA); lane 11, *C. jejuni* KLC4235 positive control; lane 12, 1kb plus marker (GibcoBRL)

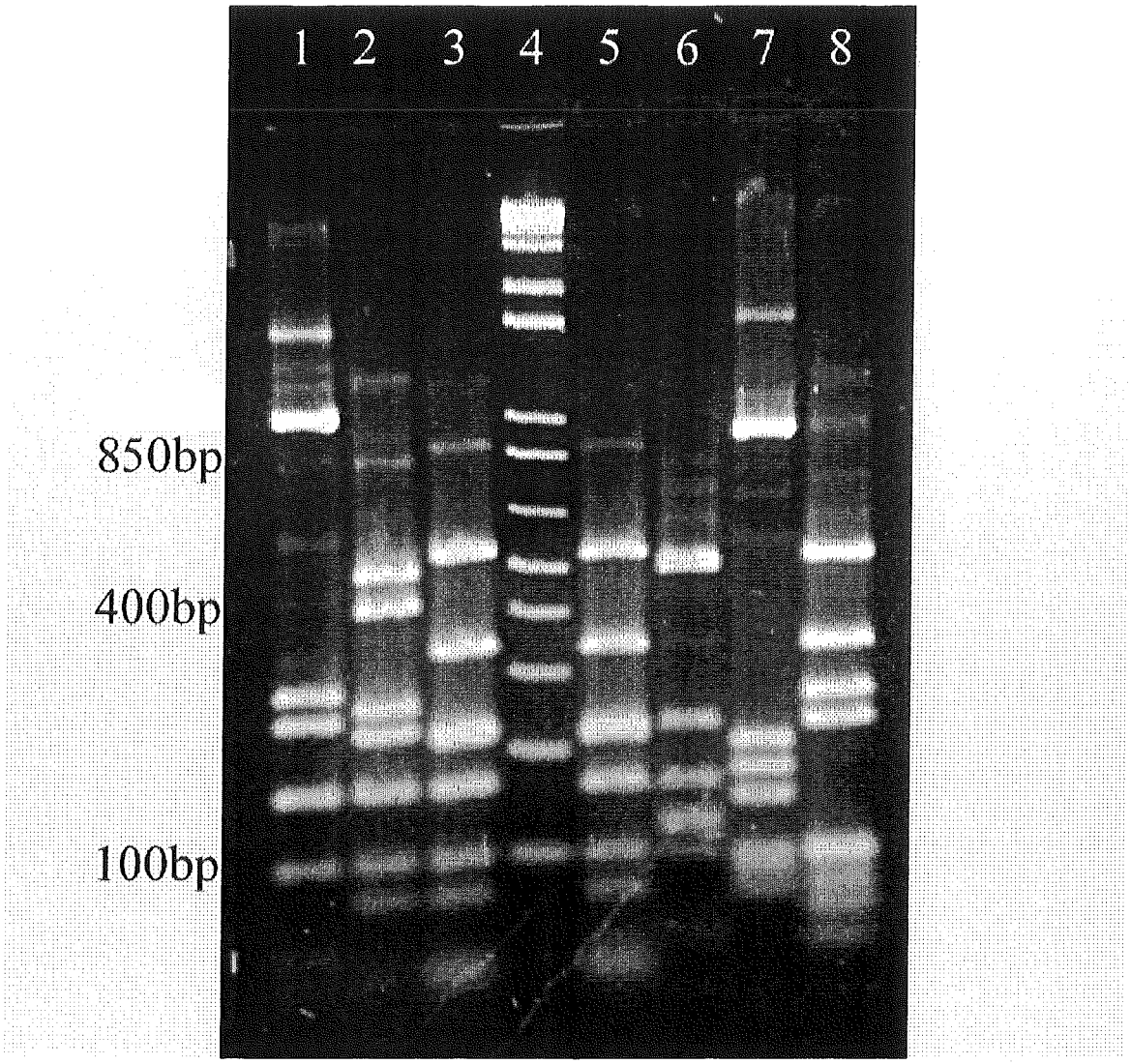

Figure 5.1b: Agarose gel of *Dde I* restriction digest profiles of the 1.7kb *flaA* amplicon generated from environmental *Campylobacter* isolates. Lane 1, HW0006; lane 2, HW0010; lane 3, HW0011; lane 4, 1kb plus marker (GibcoBRL); lane 5, HW0012; lane 6, GW0013; lane 7, GW0014; lane 8, *C. jejuni* KLC4235

Of the 17 *flaA* types observed, the largest group (*flaA 8*) consisted of three isolates. Two of these isolates were obtained from mussels placed in the Avon River while the third isolate was from a water sample taken from the Heathcote River. The next largest group consisted of two separate *flaA* types each with two isolates. *flaA 6* consisted of one water isolate from the Heathcote River and one water isolate from the Avon River. *flaA 9* consisted of two isolates obtained from Heathcote river water samples. The remaining 14 isolates each produced unique *flaA* RFLP profiles. Table 5.7 shows the *flaA* types generated by each isolate.

Table 5.7: PCR-RFLP types obtained from environmental *Campylobacter* isolates by *Dde I* digestion of 1.7kb *flaA* amplicons

Isolate	Source	*flaA* PCR-RFLP type*
AW9901	Avon river water	*flaA 1*
HW9902	Heathcote river water	*flaA 2*
SW9903	Sumner seawater	*flaA 3*
AW9904	Avon river water	*flaA 4*
AW9905	Avon river water	*flaA 5*
HW0006	Heathcote river water	*flaA 6*
AW0007	Avon river water	*flaA 6*
AW0008	Avon river water	*flaA 7*
HW0010	Heathcote river water	*flaA 8*
AM0018	Avon river mussel	*flaA 8*
AM0020	Avon river mussel	*flaA 8*
HW0011	Heathcote river water	*flaA 9*
HW0012	Heathcote river water	*flaA 9*
GW0013	Groynes water	*flaA 10*
GW0014	Groynes water	*flaA 11*
AM0015	Avon river mussel	*flaA 12*
AM0016	Avon river mussel	*flaA 13*
AM0017	Avon river mussel	*flaA 14*
AM0019	Avon river mussel	*flaA 15*
AM0021	Avon river mussel	*flaA 16*
HM0022	Heathcote river mussel	*flaA 17*

* = *flaA* type does not correspond to that of other studies

Numerical analysis of the 17 distinct *flaA* RFLP profiles obtained, allowed determination of the relationship between isolates based on *flaA* heterogeneity (Figure 5.2). The two most similar *flaA* types with a similarity coefficient of 0.7, were types 12 and 13. Both these types are represented by a single isolate; each obtained from mussels placed in the Avon River. The next most similar groups are represented by two distinct clusters, each

containing two *flaA* groups. These are *flaA* groups 4 and 16; and *flaA* groups 1 and 11, with both clusters showing a similarity coefficient of 0.6. Types 4 and 16 represent single isolates from Avon River water (AW9904) and a mussel placed in the Avon River (AM0021), respectively. Types 1 and 11 also represent single isolates obtained from an Avon River water sample and a Groynes water sample respectively. *flaA* types 3 and 7 which represent single isolates from Sumner seawater and Avon River water respectively, appear to be the most distantly related to the rest of the isolates examined.

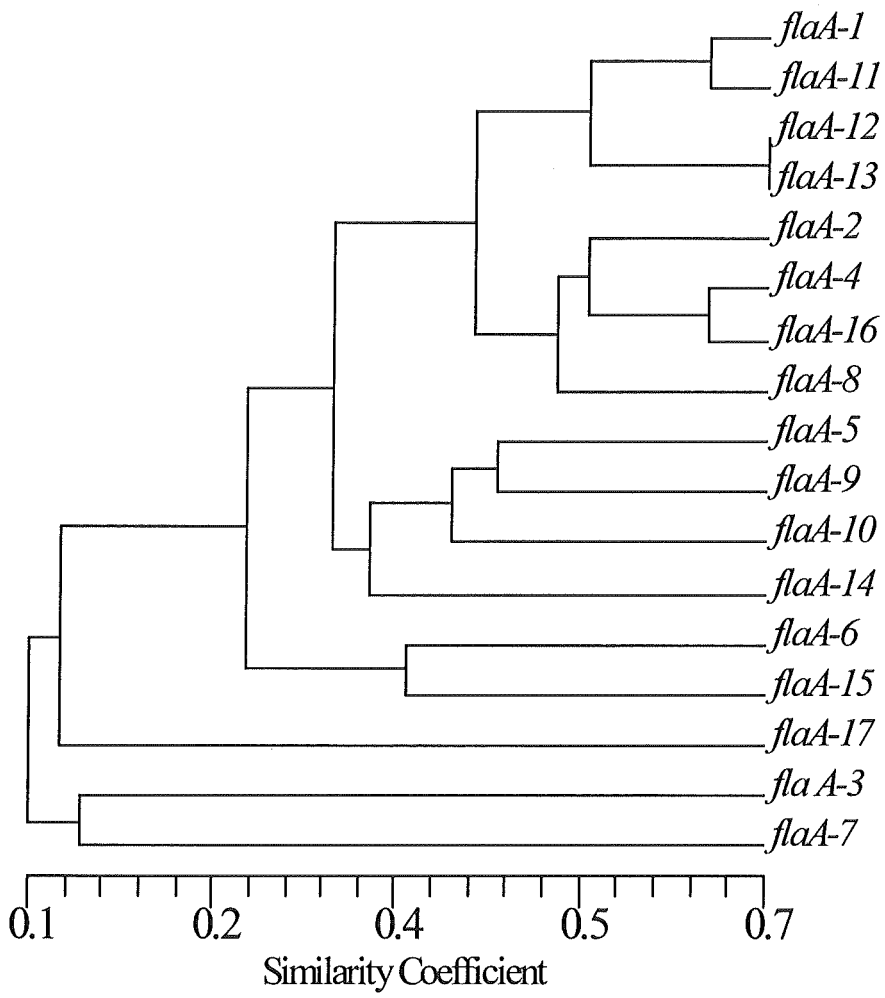

Figure 5.2: Dendrogram showing relationships between environmental *Campylobacter* isolates grouped into *flaA* types by RFLP analysis of *Dde I* digested 1.7kb *flaA* PCR amplicon

5.3.4 Pulsed-field gel electrophoresis results

Of the 22 environmental *Campylobacter* isolates analysed by PFGE, 19 (86%) generated a measurable macro-restriction profile upon digestion with the restriction endonuclease *Sma I*. The isolates that did not produce a detectable PFGE profile were isolates AM0015, AM0021, and HM0021, with repeated attempts failing to generate a restriction profile. Of the 19 isolates that produced macro-restriction profiles, 15 distinct PFGE patterns were observed. A representative sample of PFGE profiles are shown in Figure 5.3. Typical *Sma I* macro-restriction patterns consisted of between four to eight discrete bands with sizes ranging from 48 to 485kb.

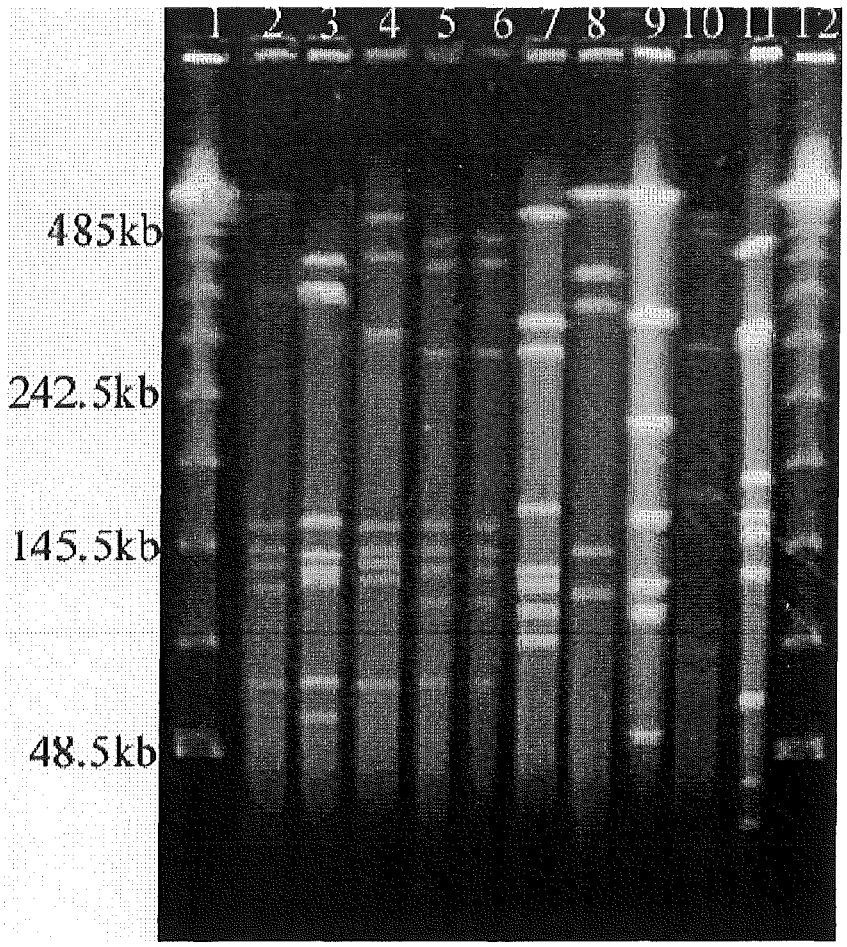

Figure 5.3: Agarose gel of *Sma I* macro-restriction digest profiles generated from environmental *Campylobacter* DNA separated by PFGE. Lane 1, Lambda ladder Pfg marker (New England Biolabs); lane 2, AW9901; lane 3, HW9902; lane 4, HW0010; lane 5, HW0011; lane 6, HW0012; lane 7, GW0014; lane 8, SW9903; lane 9, AW9904; lane 10, EC0009; lane 11, AW0008; lane 12, Lambda ladder Pfg marker (New England Biolabs)

Of the 15 PFGE types observed, the largest group (PFGE-9) consisted of three isolates. Two of these isolates were obtained from mussels placed in the Avon River with the third isolate in this group obtained from a Heathcote River water sample. The next largest groups represented PFGE types 6 and 10 each with two isolates. Isolates within group 6 consisted of a water isolate from the Avon River and a water isolate from the Heathcote River. PFGE type 10 consisted of two water isolates obtained from the Heathcote River. The remaining 12 isolates each produced unique PFGE patterns. Table 5.8 shows the specific PFGE type produced by each isolate.

Table 5.8: PFGE types obtained from environmental *Campylobacter* isolates by *Sma* I digestion of chromosomal DNA

Isolate	Source	PFGE type
AW9901	Avon river water	PFGE-1
HW9902	Heathcote river water	PFGE-2
SW9903	Sumner seawater	PFGE-3
AW9904	Avon river water	PFGE-4
AW9905	Avon river water	PFGE-5
HW0006	Heathcote river water	PFGE-6
AW0007	Avon river water	PFGE-6
AW0008	Avon river water	PFGE-7
EC0009	Estuary cockle	PFGE-8
HW0010	Heathcote river water	PFGE-9
AM0018	Avon river mussel	PFGE-9
AM0020	Avon river mussel	PFGE-9
HW0011	Heathcote river water	PFGE-10
HW0012	Heathcote river water	PFGE-10
GW0013	Groynes water	PFGE-11
GW0014	Groynes water	PFGE-12
AM0016	Avon river mussel	PFGE-13
AM0017	Avon river mussel	PFGE-14
AM0019	Avon river mussel	PFGE-15

Scoring of the presence or absence of bands at specific migration distances in relation to the PFG lambda marker resulted in a binary table that was used to generate a dendrogram showing similarity between strains grouped by PFGE type (Figure 5.4). Figure 5.4 shows that PFGE types 1 and 15 contain isolates of greatest similarity with a similarity coefficient of 0.86. PFGE-1 contains a single isolate obtained from Avon River water while PFGE-15 contains a single isolate obtained from a mussel placed in the Avon River. PFGE-2 is the next most similar isolate to the PFGE-1 and 15 cluster with a similarity coefficient of 0.71. This PFGE type consists of a single isolate (HW9902) obtained from Heathcote River water. At a similarity coefficient of 0.51, two of the largest PFGE groups (PFGE-9 and 10) are clustered together with PFGE-11. This cluster represents 40% of all isolates and consists of isolates from the Heathcote River, Avon River and the Groynes. PFGE-8 consisting of the one cockle isolate was the most distantly related to all other isolates. This is not surprising given the presumptive identification of this isolate as *C. lari*. The one *C. coli* isolate (GW0013, PFGE-11) showed a similarity coefficient of 0.61 to the three PFGE-9 *C. jejuni* isolates.

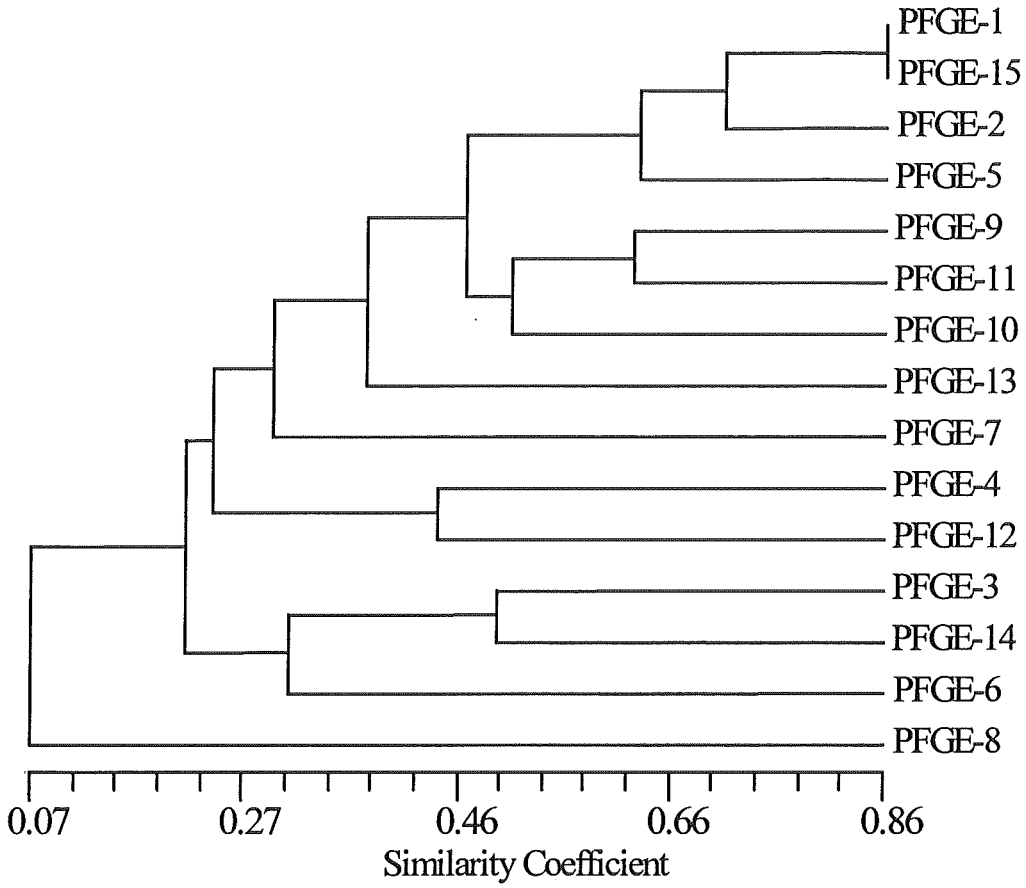

Figure 5.4: Dendrogram showing relationships between environmental *Campylobacter* isolates grouped into PFGE types by macro-restriction profile analysis of *Sma* I digested chromosomal DNA

5.3.5 Detection of *Campylobacter* virulence factors

The potential virulence of *Campylobacter* isolates was assessed by PCR detection of the *flaA*, *cadF,* and *ciaB* genes. Of the 22 isolates analysed, 21 produced the appropriate 450bp *flaA* amplicon and the 400bp *cadF* (F2B-R1B) amplicon. The one isolate that failed to produce either amplicon was EC0009 presumed to be *C. lari.* Of the 22 isolates analysed for the *ciaB* gene, 20 produced the expected 500bp amplicon. The two isolates that failed to generate an amplicon were EC0009 (*C. lari*) and GW0013 (*C. coli*). These results are shown in Table 5.9.

Table 5.9: PCR detection results of the virulence factor genes *flaA*, *cadF* and *ciaB* from environmental *Campylobacter* isolates

Isolate	Presumptive species	*flaA* amplicon	*cadF* amplicon	*ciaB* amplicon
AW9901	*C. jejuni*	+	+	+
HW9902	*C. jejuni*	+	+	+
SW9903	*C. jejuni*	+	+	+
AW9904	*C. jejuni*	+	+	+
AW9905	*C. jejuni*	+	+	+
HW0006	*C. jejuni*	+	+	+
AW0007	*C. jejuni*	+	+	+
AW0008	*C. jejuni*	+	+	+
EC0009	*C. lari*	–	–	–
HW0010	*C. jejuni*	+	+	+
HW0011	*C. jejuni*	+	+	+
HW0012	*C. jejuni*	+	+	+
GW0013	*C. coli*	+	+	–
GW0014	*C. jejuni*	+	+	+
AM0015	*C. jejuni*	+	+	+
AM0016	*C. jejuni*	+	+	+
AM0017	*C. jejuni*	+	+	+
AM0018	*C. jejuni*	+	+	+
AM0019	*C. jejuni*	+	+	+
AM0020	*C. jejuni*	+	+	+
AM0021	*C. jejuni*	+	+	+
HM0022	*C. jejuni*	+	+	+

+ = expected size amplicon produced – = no amplicon produced

Typical *flaA* amplicons were identical to those shown in Figure 3.2. Typical *cadF* amplicons were identical to those shown in Figure 4.1. Typical *ciaB* amplicons are shown in Figure 5.5.

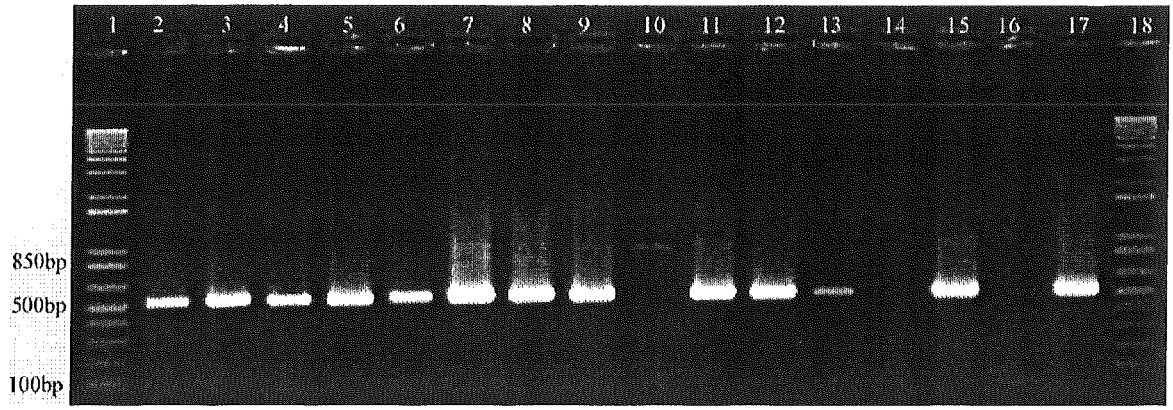

Figure 5.5: Agarose gel of *ciaB* PCR amplicons generated using the *ciaB*-F and *ciaB*-R primers with DNA from environmental *Campylobacter* isolates. Lane 1, 1kb plus marker (GibcoBRL); lane 2, AW9901; lane 3, HW9902; lane 4, SW9903; lane 5, AW9904; lane 6, AW9905; lane 7, HW0006; lane 8, AW0007, lane 9, AW0008; lane 10, EC0009; lane 11, HW0010; lane 12; HW0011; lane 13, HW0012; lane 14, GW0013; lane 15, GW0014; lane 16, Negative control (no DNA); lane 17, *C. jejuni* F38011 positive control

5.4 Discussion

5.4.1 Isolate characterisation summary

Table 5.10 summarises the results of speciation, typing and virulence determination obtained for each environmental *Campylobacter* isolate.

Table 5.10: Summary of environmental *Campylobacter spp.* characterisation

Isolate	Hip	Nal^R	Resisto-type	*flaA* type	PFGE type	*cadF* F2B-R1B	*cadF* F2B-R1C	*flaA* pg50-pg3	*ciaB*	Species
AW9901	+++	S	06	*flaA-1*	PFGE-1	+	–	+	+	*C. jejuni*
HW9902	+++	R	74	*flaA-2*	PFGE-2	+	–	+	+	*C. jejuni*
SW9903	+++	S	06	*flaA-3*	PFGE-3	+	–	+	+	*C. jejuni*
AW9904	+++	S	05	*flaA-4*	PFGE-4	+	–	+	+	*C. jejuni*
AW9905	+++	S	06	*flaA-5*	PFGE-5	+	–	+	+	*C. jejuni*
HW0006	–	S	06	*flaA-6*	PFGE-6	+	–	+	+	*C. jejuni*
AW0007	–	S	06	*flaA-6*	PFGE-6	+	–	+	+	*C. jejuni*
AW0008	++	R	26	*flaA-7*	PFGE-7	+	–	+	+	*C. jejuni*
EC0009	–	R	66	UT	PFGE-8	–	–	–	–	*C. lari*
HW0010	+++	S	06	*flaA-8*	PFGE-9	+	–	+	+	*C. jejuni*
HW0011	+++	S	04	*flaA-9*	PFGE-10	+	+*	+	+	*C. jejuni*
HW0012	+++	R	67	*flaA-9*	PFGE-10	+	+*	+	+	*C. jejuni*
GW0013	–	S	06	*flaA-10*	PFGE-11	+	+	+	–	*C. coli*
GW0014	+++	S	04	*flaA-11*	PFGE-12	+	–	+	+	*C. jejuni*
AM0015	+++	S	47	*flaA-12*	UT	+	–	+	+	*C. jejuni*
AM0016	+++	S	04	*flaA-13*	PFGE-13	+	–	+	+	*C. jejuni*
AM0017	+++	S	04	*flaA-14*	PFGE-14	+	–	+	+	*C. jejuni*
AM0018	+++	S	47	*flaA-8*	PFGE-9	+	–	+	+	*C. jejuni*
AM0019	+++	S	07	*flaA-15*	PFGE-15	+	–	+	+	*C. jejuni*
AM0020	+++	S	07	*flaA-8*	PFGE-9	+	–	+	+	*C. jejuni*
AM0021	+++	S	07	*flaA-16*	UT	+	–	+	+	*C. jejuni*
HM0022	+++	S	05	*flaA-17*	UT	+	–	+	+	*C. jejuni*

+++ = strong positive hippurate reaction ++ = positive hippurate reaction S = sensitive R = resistant 04-74 = resistotype code UT = untypeable + = positive PCR result – = negative PCR result +* = positive PCR result but non-specific bands present

5.4.2 *Campylobacter* speciation

Sodium hippurate hydrolysis and naladixic acid susceptibility tests were used for the speciation of 22 environmental *Campylobacter* isolates. These tests are readily used for distinguishing *C. jejuni*, *C. coli* and *C. lari* isolates (Bolton and Owen, 1996; Morris et al., 1985; Reed and Williams, 1998). However, a number of isolates produced conflicting results. Isolates HW9902, AW0008 and HW0012 produced strong positive hippurate hydrolysis results indicating *C. jejuni*, but were also consistently naladixic acid resistant, suggestive of *C. lari* (Table 5.10). In an attempt to confirm these isolates as *C. jejuni*, the *cadF* PCR speciation system was used. This test however, only further complicated the results. Two sodium hippurate hydrolysis negative isolates that would have been classified as *C. coli*, failed to generate a *C. coli*-specific amplicon, casting doubt as to their identity. Furthermore, two sodium hippurate hydrolysis positive isolates produced a profile of non-specific bands including a potential 450bp *C. coli*-specific *cadF* band with the F2B-R1C primer set. In chapter IV, the *cadF* PCR system was shown to be relatively reliable for speciating *C. jejuni* and *C. coli* when they had presumptively been identified using the above biochemical and physiological tests, with a high percentage of isolates producing the expected size amplicons. Hence, the conflicting results are unlikely to be due to the unreliability of the *cadF* PCR method.

In total, six isolates generated conflicting test results. A number of possible reasons could account for the conflicting results. Firstly the isolates may not have been sufficiently purified and contained mixed species of *Campylobacter*. To test this, all environmental isolates were revived from −80°C stocks and streak isolated for purification. Each isolate was passaged at least three times onto fresh CCDA taking care to pick well isolated colonies for subsequent re-streaks. Upon re-testing with the above speciation techniques, identical results were obtained. This suggests that isolates were not mixed cultures and the results were reproducible. A second possibility is that the two sodium hippurate hydrolysis negative isolates are not *C. coli* but *C. jejuni*. The presence of hippurate hydrolysis negative *C. jejuni* isolates has been previously reported (Totten et al., 1987). Totten et al. (1987) reported 20% of hippurate negative *Campylobacter* isolates to be *C. jejuni* as confirmed by genetic characterisation (spot blot test, DNA

hybridisation). Hippurate hydrolysis tests were performed by the rapid tube test method using ninhydrin as an indicator. This test, although fast and simple is dependent on inoculum size (On and Holmes, 1991) and hence is not as reliable as more complex methods. Morris et al. (1985) tested four hippurate hydrolysis methods for identification of *C. jejuni* isolates. Hippurate hydrolysis by the gas-liquid chromatography (GLC) procedure was found to be more sensitive than three tube methods. Using GLC, 100% of *C. jejuni* isolates were positive for hippurate hydrolysis while three strains were consistently negative with tube tests (Morris et al., 1985). Totten et al. (1987) reported a similar finding. Hence it is possible that the hippurate negative isolates HW0006 and AW0007 are in fact *C. jejuni*. The *cadF* PCR result supports this. Use of the *hip* PCR assay to specifically detect the hippuricase gene from these isolates would confirm the presence or absence of this gene from these isolates.

Three isolates were naladixic acid resistant but gave strong hippurate positive tests. It is unlikely that these strains are *C. lari* as it is not uncommon for bacteria to acquire antibiotic resistance genes through interspecies transfer of DNA in certain environmental conditions (Lucey et al., 2000). *Campylobacter* species in particular are known to possess a natural ability for transformation (Lucey et al., 2000). Furthermore, the presence of naladixic acid resistant *C. jejuni* have been reported (Reed and Williams, 1998). As hippurate hydrolysis positive *C. coli* and *C. lari* isolates are highly uncommon, the observation of a strong positive hippurate hydrolysis result was considered to be the most reliable result and hence these isolates were presumptively classified as *C. jejuni* regardless of resistance to naladixic acid. The current limited number of tests for the phenotypic characterisation, each with subjective interpretations is a major hindrance to the identification of *Campylobacter spp.* (Bolton and Owen, 1996). A need to establish phenotypic and non-phenotypic criteria for identification of *Campylobacter* is apparent.

5.4.3 Resistotyping

Resistotyping was shown to have a high level of typeability with all isolates producing a result. This has also been demonstrated by previous studies (Ribeiro et al., 1996;

Armstrong, 1997; Calder, 1998). Reproducibility was also high when duplicate tests were performed simultaneously. However, when duplicate tests for each isolate were performed on different occasions, the resistotype pattern was not always reproducible. A number of possibilities could account for this. These include the inoculum size used for obtaining a bacterial lawn, the length of incubation before results were analysed and the induced resistance to one or more of the antibiotics used. The most likely factor however, is the subjective interpretation of individual results which may in part be due to the above three factors. Owen et al. (1997) also observed this lack of reproducibility with this resistotype scheme. A total of 51% of *C. jejuni* strains tested and 36% of *C. coli* strains tested produced a different resistotype code upon re-testing (Owen et al., 1997). Owen et al. (1997) also observed that incubation times of 24 and 48h resulted in different resistotype codes from the same isolates. Similarly, five strains that were not typeable due to poor growth and hazy zones of inhibition, gave readable results when a higher inoculum was used. In the current study, duplicate tests performed simultaneously would have had very similar inocula, incubation conditions and identical incubation times. When repeat tests were performed separately, variations in these biochemical and environmental conditions could have resulted in the differing resistotype codes. Furthermore, differences in the interpretation of similar results may also account, in part, for the lack of reproducibility. Dependence on the prevailing environmental conditions for the expression or non-expression of the phenotype tested is a major limitation of most of most phenotypic typing methods and hence results in the preference for the more stable genotypic typing methods.

In this study, the three most frequent resistotypes encountered were, in descending order, 06, 04, and 07 accounting for 63.8% of all isolates tested (Table 5.10). In previous local studies by Armstrong (1997) and Calder (1998), the most common resistotypes reported were 04, 01, 00 and 02, 16, 06 respectively. In the study by Armstrong (1997) resistotype 04 accounted for approximately 25% of all (mostly clinical) isolates tested. This compares with a similar percentage (18.2%) of environmental isolates generating this resistotype in the current study. Interestingly, resistotype 04 was not encountered by Calder (1998) who tested a range of locally isolated *Campylobacter* strains from birds

and water. International studies by Ribeiro et al. (1996) and Owen et al. (1997) most frequently encountered resistotypes of 40, 02, 00 and 40, 00 respectively. These differences are consistent with the expectation that that localised selective pressures result in the variation in resistance patterns expressed between isolates from geographically distinct regions. Resistotypes from the three sampling sites from which more than one isolate was obtained did not always group together with isolates from the Avon River, Heathcote River, the Groynes and Sumner seawater being grouped together in the largest resistotype group (06). This might suggest that, taken alone, a common source of *Campylobacter* input exists between all of these sites (although genetic data below do not appear to support this). The most likely candidates are the birds (mostly ducks and gulls) that are common to each of the sites. One of the most pronounced results observed was the 100% resistance of isolates to sodium arsenite (NaAr). This resistance level is much higher than reported from all previous studies. However, high levels of NaAr resistance were also observed by Armstrong (1997) and Calder (1998) with resistances of 60% and 36% respectively. It is possible that the two to three year difference between these studies and the current study has resulted in an increasing number of isolates acquiring NaAr resistance. These high numbers of NaAr resistant isolates has been postulated to be due to high levels of NaAr in Canterbury soils (Calder, 1998). Studies by Ribeiro et al. (1996) and Owen et al. (1997) have reported much lower NaAr resistances for *Campylobacter* isolates in the UK. Although a 60% NaAr resistance was reported by Armstrong (1997), 100% of isolates obtained from water were resistant to this antimicrobial agent.

Analysis of isolate similarity based on resistotypes showed that the majority of isolates were 50% similar (Appendix IV, figure A4.1) with a high level of similarity between isolates from the Avon, Heathcote Rivers and the Groynes. Although resistotyping was rapid and simple to perform, problems with reproducibility make the scheme somewhat unreliable. Furthermore, this system was also found to group apparently unrelated organisms within the same resistotype (Owen et al., 1997; see also *flaA* and PFGE typing results below). Hence, resistotyping can show that isolates are distinct but cannot unequivocally confirm that isolates are identical (Ribeiro et al., 1996).

5.4.4 *flaA*-RFLP analysis

All isolates that were presumptively classified as *C. jejuni* or *C. coli* were typeable using the *flaA* RFLP method. The one isolate that failed to generate a 1.7kb *flaA* amplicon is presumed to be a *C. lari* and as such, was not expected to produce an amplicon specific for *C. jejuni* and *C. coli* isolates. The high level of typeability (100% for *C. jejuni* and *C. coli* strains) is much higher than previous studies. Calder (1998) reported only 45% of environmental isolates typeable while Armstrong (1997) reported 60% of environmental isolates were typeable by *flaA* RFLP. Nachamkin et al. (1993) who developed the *flaA* typing system used in this study, found approximately 83% of both clinical and environmental (cows, pigs, birds) isolates were typeable. The major difference between the methods used by the above three studies and the current study was the type of DNA template used for PCR amplification. All three previous studies used whole cell lysates prepared by boiling cells to release DNA. In the current study, purified chromosomal DNA was used for *flaA* typing and is likely to have resulted in the higher level of typeability. Mohran et al. (1998) has shown that some *Campylobacter* strains are resistant to lysis in boiling water and do not release intact high molecular weight DNA upon whole cell lysate preparation. Hence, the use of purified DNA is preferable when using PCR-based typing methodologies.

A total of 17 different restriction patterns were observed by *flaA* RFLP (Table 5.10). *FlaA* typing can be considered to be twice as discriminatory as resistotyping, as only eight resistotype groups were identified among the same isolates. This shows that the environmental *Campylobacter* isolates are genetically diverse with respect to the *flaA* gene. Only three *flaA* groups contained more than one isolate. Single isolates from the Avon River and Heathcote River were contained within two of these *flaA* types, (*flaA-8* and *16*). This gives further evidence that both rivers have a common source of *Campylobacter* input. While the largest resistotype group (06) contained seven isolates, only two were grouped within the same *flaA* type, with all five other isolates producing unique *flaA* profiles. This confirms that resistotyping cannot demonstrate isolates to be identical. Such a high level of *flaA* gene heterogeneity among *Campylobacter* isolates was also observed by Calder, (1998) who reported 12 distinct profiles from 17

environmental isolates. In *flaA* typing studies where large numbers of clinical isolates were analysed, the gene heterogeneity was not as diverse. Armstrong (1997) observed 20 RFLP patterns from 43 mostly clinical isolates while Nachamkin et al. (1993) observed 18 RFLP profiles from 43 isolates. Koenraad et al. (1995a) reported only 22 different RFLP patterns for 182 isolates from sewage plants and wastewater, while Nachamkin et al. (1996) reported 83 flagellin types from 404 largely clinical isolates. Nachamkin et al. (1996) found six of these 83 RFLP types accounted for 53.6% of all strains tested. In the current study, the three largest *flaA* groups only accounted for 33% of isolates tested.

Based on the genetic relatedness of *flaA*, isolates from the Avon River were found to be the most similar, with the Groynes *C. coli* isolate (GW0013) showing the greatest level of similarity to these closely related Avon River isolates. Isolates from the Heathcote River were often found to show a greater level of similarity and hence genetic relatedness to Avon River isolates than to each other. The differences in interlaboratory variation, analysis methods of results and gel mobility differences meant that the *flaA* typing results obtained in this study could not be directly compared to that of other studies. Meinersmann et al. (1997) has suggested that a DNA sequence-based method for sub-typing may be more reliable in the face of these limitations. Another potential problem with *flaA* typing is the reported recombination within the *fla* locus between different strains of *Campylobacter* in natural populations (Harrington et al., 1997). Intergenomic (and intragenomic) recombination results in alterations to the gene sequence while the gene function itself remains unchanged. Therefore, relationships amongst *Campylobacter* strains as a result of flagellin gene typing may not accurately reflect true clonal relationships between strains as determined by their overall genetic similarity (Harrington et al., 1997). Therefore, analysis of several different genes or use of PFGE techniques is preferred to assess constant genetic relationships amongst isolates.

5.4.5 PFGE typing

In theory, all *Campylobacter* isolates should be typeable by PFGE due to the fact that the presence or absence of a gene is not a factor and all bacterial cells consist of genomic DNA. In reality, a few strains are occasionally reported as untypeable. In the current

study, only 19 (86%) of 22 isolates were typeable. Two major reasons could explain the untypeability of three isolates. The first possibility is that the restriction endonuclease *Sma* I was unable to cut the DNA from these three isolates. Considering that *Sma* I is a widely used enzyme for PFGE analysis of *Campylobacter* genomes (Hanninen et al., 1998a; On et al, 1998; Steele et al., 1998; Wassenaar et al., 1998), this is unlikely. However, Hudson et al. (1999) reported that 25 of 163 isolates were in fact not typeable due to the failure of the *SmaI* enzyme to cut DNA. The second possibility is that these isolates are DNase positive, thus degrade their genomic DNA during the agarose plug preparation process. This is more likely to be the case as when plugs were re-prepared using an increased cell turbidity of McFarland #3, no DNA pattern was observed on the resultant gel. The degradation of *C. jejuni* chromosomal DNA during standard PFGE preparative procedures has been previously reported (Santesteban et al., 1996-cited in Calder, 1998).

Of the 19 strains that were typeable, 15 distinct PFGE patterns were produced. The cockle isolate (EC0009) that was not typeable by *flaA* RFLP was typeable by PFGE. If the three strains that failed to generate a PFGE profile and the one strain that was *flaA* non-typeable are removed, a total of 14 distinct groups are observed by both genetic typing methods. Of these 14 types, the groupings by PFGE and *flaA* RFLP typing match each other exactly. For example, the three isolates grouped within PFGE group 9 are all in *flaA* type 8. Similarly, all isolates that produced a distinct *flaA* type also produced distinct PFGE types. Such a high level of identity between these two typing methods has not been observed by all researchers. Wassenaar et al. (1998) reported 21 single colony isolates of *C. jejuni* that produced an identical flagellin genotype, generated 14 distinct PFGE profiles. It is possible that water isolates may have a greater degree of genomic stability than those reported from poultry by Wassenaar et al. (1998). Although PFGE has been reported to have a much higher discriminatory power than *flaA* typing, other studies have reported much less diversity amongst *Campylobacter* isolates than the 15 types from 19 strains reported in this study. On et al. (1998) reported six *SmaI* profiles from a diverse group of *C. jejuni* isolates obtained from humans, water, poultry and cattle. Steele et al. (1998) reported 86 different *Sma* I profiles from 141 *Campylobacter*

strains from a meat processing plant while Hanninen et al. (1998b) found 69 PFGE types among 176 clinical isolates. In contrast to the relatedness observed by *flaA* typing, analysis of relatedness based on the presence or absence of *Sma* I digested DNA bands showed marked differences. Similar to *flaA* typing, PFGE analysis showed most Avon River isolates to be closely related to each other with many Heathcote River isolates more closely related to Avon River isolates than to each other. However, PFGE deemed PFGE type 1 and 15 to be the most genetically related with a similarity coefficient of 0.86. These types represent isolates AW9901 and AM0019 respectively. *flaA* typing deemed these isolates to be only approximately 24% similar. Conversely, isolates AW9901 and GW0014 were found to be 60% similar by *flaA* typing while PFGE typing deemed these isolates to be distantly related. As such, the PFGE results are most likely to represent the most accurate estimate of genetic relatedness as the whole bacterial chromosome is taken into account as opposed to a single gene.

From the evidence generated by PFGE and *flaA* typing, it may be assumed that isolates HW0010, AM0018 and AM0020 are clonal (PFGE-9 and *flaA-6*) as are isolates HW0006 and AW0007 (PFGE-6 and *flaA-6*) and HW0011 and HW0012 (PFGE-10 and *flaA-9*). As previously noted, the clonality of isolates obtained from the Avon and Heathcote Rivers is highly indicative of a common input source. The major likelihood is that *Campylobacter spp.* are entering the rivers via stormwater drains or by point sources from the large bird populations common to each river. PFGE, although time consuming, is an invaluable epidemiological tool for assessment of *Campylobacter* sources and genetic relatedness.

Of the three typing methods used in this study, resistotyping was found to be the least discriminatory with only nine resistotypes generated. The largest resistotype group (06) containing seven isolates could be further divided into 6 distinct groups by both *flaA* and PFGE. Both of these genetic methods showed an identical level of discrimination. The advantages of using both these methods meant that isolates that were unable to be typed by one method, could be by the other, and allowed for the assurance of results. The testing of a greater number of isolates from water and birds within the Avon, Heathcote

Rivers and estuary as well as the Groynes would give useful insights into the sources and diversity of *Campylobacter* inputs to these recreational water supplies.

5.4.6 Assessment of potential virulence

The detection of the virulence factor genes *flaA, cadF* and *ciaB* was a rapid and simple way to assess the potential virulence of environmental isolates. If environmental *Campylobacter* isolates do not have the necessary genes for causing disease in humans, they pose no risk to public health. Therefore the assessment of potential pathogenicity in environmental isolates was essential for implicating local recreational water supplies as possible vehicles for *Campylobacter* transmission. Each of the genes tested encodes a protein important for specific steps during pathogenesis, namely motility, adherence and invasion. All isolates tested except one produced a *cadF* and *flaA* PCR amplicon. This suggests that each isolate has the potential for swimming through the intestinal mucosal layer and adhering to epithelial cells should such conditions be encountered. The *C. lari* isolate failed to generate any amplicon with these primers. As *C. lari* isolates are rarely associated with disease (Simor and Wilcox, 1987; Skirrow, 1990), this is not surprising, although it was expected to have the *flaA* gene. All environmental *Campylobacter* isolates produced a *ciaB* PCR amplicon except the *C. lari* isolate and the *C. coli* isolate. Given that a *C. coli* isolate was not assayed for binding and internalisation by Konkel et al. (1999b), whether or not *C. coli* possesses *ciaB* is unclear. This strain may be able to cause disease by toxin production as has been suggested for some *Campylobacter* species (Misawa et al., 1994; Misawa et al., 1995), but lacks the ability to cause invasive disease. All other isolates can be assumed to have the potential to cause invasive disease.

As the potential for pathogenicity does not necessarily mean the organism will definitely cause disease when given the opportunity, tissue culture assays can be used to assess virulence. Tissue culture assays have successfully been used for assessment of pathogenic properties by a number of workers. Harvey et al. (1999) demonstrated ten different *Campylobacter* isolates to have variable invasion phenotypes upon culture with Caco-2 cells. Hence it is likely that the 22 environmental isolates obtained in the current study would show different levels of disease severity despite most having the necessary

genes for pathogenesis. Time constraints and a lack of facilities for tissue culture assay did not allow these experiments to be performed on environmental isolates. Hence, although the potential for pathogenicity is present, the actual virulence of these isolates cannot be inferred.

Chapter VI

Summary of Results and Conclusions

This study was undertaken with the premise of evaluating the suitability of aquatic invertebrates to act as a gauge in a novel, alternative method to undependable water sampling procedures for the detection of *Campylobacter* species from recreational water supplies. In order to do this, an environmental sampling program was undertaken whereby the incidence of *Campylobacter* species in a range of recreational water types and naturally occurring aquatic invertebrates (cockles, mussels, snails) were assessed as invertebrate biological indicators. The lack of readily available macro-invertebrates in local rivers led to the placement of freshwater mussels in the Avon and Heathcote Rivers. *Campylobacter* isolates recovered from sampling were speciated and characterised by phenotypic and molecular typing methods to determine possible links between differing sources and assess genetic relatedness. The freshwater mussel was assessed for its appropriateness as a biological indicator by examining the uptake and accumulation of *C. jejuni* and comparing it with the survival and detection of *C. jejuni* from freshwater. As a marker for determining potential virulence, the detection of the *cadF* virulence gene in a PCR detection system was utilised and validated.

Prior to environmental sampling, the sensitivity of two *Campylobacter* culture detection methods (passive filtration and enrichment with Exeter broth), were tested. The filter enrichment method was found to be the most sensitive, able to detect an initial input inoculum of <2 cfu. This method was used for all subsequent environmental sample processing. The recovery of *Campylobacter* isolates from aquatic environments was low. A total of 22 isolates were obtained, the majority of which were isolated from freshwater. Extremely low isolation rates were observed amongst the three naturally occurring invertebrates tested (cockles, snails, mussels). Only one isolate was recovered from a cockle while mussels and snails did not harbour culturable *Campylobacter spp.* Of the

limited number of *Campylobacter* isolates obtained by testing invertebrates and the surrounding water, there were no strong correlations between the presence of *Campylobacter* in the water and presence of *Campylobacter* in the invertebrates tested. It was concluded that *Campylobacter spp.* are highly variable in their presence or absence within the marine waters containing invertebrates, therefore correlations between existence in water and in naturally occurring fauna could not be made. Future studies could include a long term environmental sampling study encompassing the testing of invertebrates, water and birds from in and around the Avon-Heathcote Rivers and estuary to generate a comprehensive indication of prevalence, transmission, cycling, seasonal fluctuations and true risks to public health from recreational use of this local and highly accessible aquatic system as well as from consumption of shellfish.

The use of freshwater mussels for isolating *C. jejuni* from rivers was successfully applied in this study. A total of eight isolates were successfully purified and *Campylobacter* was detected by PCR in a further 16 mussels. This amounted to a detection rate of 60% (24 positive from 40 mussels), which was a 25% higher detection rate than from the surrounding water. These high detection rates from freshwater mussels and the very low isolation rates from other invertebrates tested made the freshwater mussel the logical choice for further assessment as a possible bio-indicator. Comparison of *C. jejuni* accumulation and detection in the mussel with *C. jejuni* survival and detection in water found a close correlation in detection time lengths. *C. jejuni* was detected in up to 100% of water and mussel samples taken at 96h post-inoculation. All samples taken beyond 96h were negative from mussels and water with the exception of one mussel taken at 3 weeks post-inoculation. This mussel was considered an outlier and probably does not reflect the proliferation or survival of *C. jejuni* in mussels. At a lower inoculation concentration, *C. jejuni* was detectable from water and mussels for a shorter duration but still very closely matched each other in detection time lengths. The survival of *C. jejuni* in water was assessed by direct culturable counts from seeded water. At 15°C cells showed an initial decline in numbers followed by a transient increases in culturable cell numbers at four and 12 hours post inoculation. By 72h cells had lost culturability as measured by culturable counts but were still detectable by enrichment culture for a

further 48h. These transient increases are not an uncommon phenomenon and have been observed by other researchers (Calder, 1998; Ekweozer et al., 1998). Assessment of the freshwater mussel to be a suitable bio-indicator was judged by five specific criteria. The *C. jejuni* uptake and detection results indicated that the mussel met four of these five criteria. The only criterion that was not met was 'the ability to maintain cells in a culturable form for longer periods than the surrounding water'. This however, was not assessed in detail by the uptake experiments. It was concluded that although detection of *C. jejuni* from mussels was as good as detection from water, it was no better than testing the water itself by enrichments. Therefore, under normal circumstances, use of the Exeter enrichment broth method is adequate for testing the water for *Campylobacter spp.* Future research could more closely address the VNC state and resuscitation or maintenance of culturability within mussels. Direct inoculation of water induced VNC cells into mussel digestive tracts and the subsequent analysis of faeces, pseudofaeces and flesh for culturable cells may provide useful insights into bacterial dynamics within shellfish. Similarly, if mussels can maintain VNC cells at high concentrations, an increased risk of gastroenteritis due to shellfish consumption could be elucidated.

Factors influencing the growth and genetic stability of *C. jejuni* present in the mussel and water environments were evaluated. The possible inhibitory effects of mussel flesh in Exeter enrichment broth was tested by comparing *C. jejuni* growth on Exeter agar with Exeter agar containing mussel flesh. Mussel flesh agar was completely inhibitory to the growth of *C. jejuni* on solid media. Obvious differences in growth conditions exist between solid and liquid media which allowed the proliferation of *C. jejuni* in an inhibitory environment. Recovered *C. jejuni* after passage into mussel flesh and from water were tested for genetic changes in the *flaA* gene as compared to the wild type strain. PCR-RFLP analysis showed the *C. jejuni* test strain to exhibit genetic stability of the *flaA* gene in both the water and mussel environments. An interesting experiment for future research could address the role of virulence factors in the environmental survival of *Campylobacter* strains. By performing water detection and invertebrate uptake experiments on virulence factor minus isolates, valuable insight could be gained as to the

evolution of virulence factors as specific for pathogenesis or as important environmental survival determinants with pathogenesis properties as purely coincidental.

Campylobacter species were not detected from sampling of marine mussels or snails. Marine mussels and cockles have previously been shown to rapidly accumulate *C. jejuni* in water when present (Calder, 1998). Estuarine snails however, have not been studied. To determine whether estuarine snails were capable of accumulating *C. jejuni* when present in the water, tanks containing snails were inoculated with *C. jejuni* and periodically sampled for culturable cells. Snails rapidly accumulated *C. jejuni* from water but were efficient at eliminating cells from their tissues. At low inoculation concentrations, detectability from snails was lost within one hour with a subsequent regain in culturability at four hours post-inoculation. At high concentrations, *C. jejuni* was detectable in snails for up to 72h. Within the Avon-Heathcote estuary, it was suspected that snails rapidly degraded any *Campylobacter* cells accumulated from water or sediments and hence the lack of *Campylobacter* isolation.

The specificity and discriminatory power of the *cadF* PCR speciation system was tested in a *cadF* validation study. A total of 220 bacterial isolates comprising 134 *C. jejuni* isolates, 18 *C. coli* isolates, six *C. lari* isolates, 13 other *Campylobacter* species and 49 non-*Campylobacter* isolates were tested for the *cadF* gene. Using the primers F2B and R1B, 99% of *C. jejuni* and 95% of *C. coli* isolates generated the expected size amplicon. In addition, 100% of presumed *C. lari* isolates and three other *Campylobacter* isolates (*C. hyoilei, C. hyointestinalis, C. rectus*) produced the amplicon specific for *C. jejuni* and *C. coli* isolates. Furthermore, two non-*Campylobacter* strains (*Branhamella cattharalis* and *Listeria innocua-B*) also produced the 400bp amplicon. Although amplicons produced by the non-*C. jejuni* or *C. coli* isolates were not confirmed as *cadF* or otherwise, it was presumed that most of these were non-specific bands. A logical next step is to confirm whether these amplicons are a true *cadF* amplicon by Southern bolt or sequencing. Use of the F2B-R1C primers specific for *C. coli* showed highly variable results. Only 67% of *C. coli* isolates produced the expected amplicon while a number of *C. jejuni, C. lari,* other *Campylobacter spp.* and non-*Campylobacter* species produced

non-specific bands, some of which were comparable to the expected 450bp product. It was concluded that the *cadF* gene was ideally suited for determining virulence potential in pure cultures of *C. jejuni* or *C. coli*, but showed a degree of unreliability for specific detection from environmental samples where an excess of non-target DNA may be present. Future work with *cadF* PCR should address the presence of the *cadF* gene and protein amongst confirmed *C. lari* isolates and attempt to confirm by sequencing, whether other isolates also contain the gene sequence.

Speciation and characterisation of environmental isolates provided important information on the similarity between isolates and suggested a possible common source. Speciation revealed the majority of isolates obtained to be *C. jejuni* with one *C. lari* and one *C. coli* isolate presumptively identified. Resistotyping was used as a phenotypic typing method and grouped the 22 isolates into nine resistotypes. When compared to genetic typing techniques used, this method was found to be the least discriminatory. PCR-RFLP of the *flaA* gene and PFGE were found to be highly discriminatory with *flaA* typing revealing 17 distinct groups from 21 typeable isolates while PFGE generated 15 distinct types from 19 typeable isolates. Cluster analysis of PFGE and *flaA* groups revealed a high level of genetic relatedness amongst freshwater isolates from the Avon river, the Heathcote river and the Groynes, suggesting a common source of input believed to be the bird populations common to these sites. The potential virulence of environmental isolates was evaluated by PCR detection of specific virulence factor genes. All *C. jejuni* isolates were positive for the *flaA* and *cadF* and *ciaB* amplicons. The *C. coli* isolate was positive for the *flaA* and *cadF* genes but negative for *ciaB* while the *C. lari* isolate was negative for all three virulence genes. The potential for all *C. jejuni* and *C. coli* isolates analysed to be pathogenic if given the opportunity was inferred from these results suggesting a potential health risk to recreational water users. Future studies with these isolates could include tissue culture assays to determine the possible severity and mechanisms of pathogenesis.

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Appendix I

Media

I.i General media

Unless otherwise stated, media were sterilised by autoclaving for 20min at 103.4kPa at 121°C. Solutions that were unstable at this high temperature were filter sterilised by passing through a 0.22μm filter and added to sterile media.

Campylobacter Blood-Free Selective Agar Base (Modified CCDA-Preston) (Oxoid)

Typical Formula	Per litre
Beef extract	10g
Peptone	10g
NaCl	5g
Charcoal	4g
Casein hydrolysate	3g
Sodium deoxycholate	1g
Ferrous sulphate	0.25g
Sodium pyruvate	0.25g
Bacteriological agar	12g

22.75g of *Campylobacter* Blood-Free Selective Agar Base was suspended in 500ml of dH_2O. pH was adjusted to 7.4 ± 0.2 followed by autoclaving. Media was cooled to 50°C before addition of 3.2mg/L (final concentration) of cefoperazone. The agar was mixed well and poured into sterile petrie dishes.

Solution A

	Per 50ml
Ferrous sulphate	1.25g
Sodium metabisulphite	1.25g
Sodium pyruvate	1.25g

Ferrous sulphate was dissolved in 50ml of dH_2O followed by sodium pyruvate and metabisulphite. Solution was sterilised by filter sterilisation. 10ml/L of solution A was added to sterile media.

Supplement B

	Per 100ml
Rifampicin	50mg
Trimethoprim	50mg
Polymixin B sulphate	25000iu (3.2mg)
Cyclohexamide	500mg

Rifampicin was dissolved in 10ml methanol. Trimethoprim was dissolved in 90ml ddH_2O with addition of a few drops of 8.5% lactic acid. The rifampicin and trimethoprim solutions were combined followed by addition of the polymixin B and cyclohexamide and thorough mixing. Supplement B was sterilised by passing through a 0.22μm filter. 20ml/L of supplement B was added to sterile media.

Exeter Enrichment Broth

Typical formula	per litre
Oxoid Nutrient Broth #2	25g
Beef extract 10g	
Peptone 10g	
NaCl 5g	
Defibrinated sheep blood	50ml
Solution A	10ml
Supplement B	20ml

12.5g of NB #2 was added to 460ml dH_2O, mixed thoroughly and sterilised by autoclaving. Broth was cooled to 50°C before addition of defibrinated sheep blood, solution A and supplement B. Broth was mixed thoroughly and stored at 4°C when not in use.

Exeter Agar

Exeter agar was prepared as described above with the addition of 7.5g of bacteriological agar per 500ml of nutrient broth #2 prior to autoclaving. Molten agar base was cooled to 50°C before addition of defibrinated sheep blood, solution A and supplement B. Agar was mixed thoroughly and poured into Petrie dishes.

Mueller Hinton Blood Agar

Typical Formula	Per litre
Beef infusion	30g
Acid digest of casein	17.5g
Starch	1.5g
Bacteriological agar	17g
Defibrinated sheep blood	50ml

19g of the agar base was mixed with 475ml dH_2O and sterilised by autoclaving. Agar was cooled to 50°C before addition of defibrinated sheep blood, thorough mixing and dispensing into sterile petrie dishes.

Brain Heart Infusion Broth

Typical formula	Per litre
Beef heart infusion	25g
Calf brain infusion	20g
Protease peptone	10g
NaCl	5g
Na_2HPO_4:$12H_2O$	2.5g
Glucose	2g

19.0g of broth was mixed with 500ml dH_2O and sterilised by autoclaving

Bold's Basal Medium (BBM)

Typical formula (Stock solutions)

	Per litre
1. $NaNO_3$	25g
2. $MgSO_4.7H_2O$	7.5g
3. NaCl	2.5g
4. K_2HPO4	7.5g
5. KH_2PO_4	17.5g
6. $CaCl_2.2H_2O$	2.5g
7. $ZnSO_4$	8.8g
$MnCl_2.H_2O$	1.4g
MoO_3	0.71g
$CuSO_4.5H_2O$	1.5g
$CuNO_3.6H_2O$	0.49g
8. H_3BO_3	11.42g
9. EDTA	50g
KOH	31g
10. $FeSO_4.7H_2O$	4.98g
H_2SO_4 (conc)	1.0ml

Solutions 1-10 each made up to 1L in dH_2O.

Final medium made by combining 10ml of stock solutions 1 to 6 and 1ml stock solutions 7 to 10 and made up to 1L with dH_2O

Appendix II

Buffers And Solutions

II.i Common buffers & solutions

Buffers and solutions requiring sterilisation were autoclaved for 20min at 121°C at 103.4kPa or filter sterilised by passing through a 0.22μm filter. Unless stated otherwise, all buffers and solutions were stored at ambient temperature.

50 × TAE

	Per litre
50mM Tris base	242g
0.11% v/v glacial acetic acid	57.1ml
1mM EDTA (pH 8.0)	46.5g

Made up to 1L in dH_2O and pH adjusted to 8.0

1 × TAE

20ml of 50 × TAE made up to 1L with dH_2O

6 × DNA Loading Dye for Agarose Gel Electrophoresis

50% (v/v) Glycerol

0.25% (w/v) Bromophenol blue

0.25% (w/v) Xylene cyanol

Made up to 10ml in dH_2O

Phosphate Buffered Saline

	Per litre
NaCl	8.5g
KH_2PO_4	0.3g
Na_2HPO_4	0.6g

Dissolved in dH2O to a final volume of 1l, pH adjusted to 7.4.

Ninhydrin Reagent

	Per 100ml
Ninhydrin	3.5g

Dissolved into a 1:1 mixture of acetone and butanol

II.ii Chromosomal DNA Purification Solutions

Set Buffer

75mM NaCl

25mM EDTA

20mM Tris-HCl

Prepared by appropriately diluting stock solutions to the above final concentration using dH_2O

10% Sodium Dodecyl Sulphate (SDS)

	Per litre
SDS	100g

Dissolved in dH_2O at 68°C to a final volume of 1L. pH was adjusted to 7.2 with concentrated hydrochloric acid

5M Sodium Chloride

	Per litre
NaCl	295g

Dissolved in 800ml dH_2O by heating. Made up to 1L final volume.

Phenol:Chloroform:Isoamyl Alcohol

	Per 50ml
Tris Buffered Phenol	25ml
Chloroform	24ml
Isoamyl Alcohol	1ml

Mixed thoroughly and stored at 4°C in a dark glass bottle

3M Sodium Acetate

	Per litre
NaAc:$3H_2O$	408g

Dissolved in dH_2O to a final volume of 1L, pH adjusted to 5.2 with glacial acetic acid

1 × TE Buffer

10mM Tris-Cl

1mM EDTA

50ml of 20 × TE buffer (see Appendix III) made up to 1L with dH_2O

GES Lysis Solution

	Per 100ml
Guanidium thiocyanate	60g
0.5M EDTA, pH 8.0 (see Appendix III)	20ml
ddH_2O	20ml
10% v/v N-lauroyl sarcosine	5ml

Guanidium thiocyanate and ddH_2O heated at 65°C and mixed until dissolved. 10% N-lauroyl sarcosine added after cooling. Made up to 100ml with ddH_2O and passed through a 0.45μm filter

7.5M Ammonium Acetate

	Per litre
NH_4Ac	577.5g

Dissolved in dH_2O to a final volume of 1L

Chloroform:isoamyl alcohol

	Per 50ml
Chloroform	48ml
Isoamyl alcohol	2ml

Mixed thoroughly and stored at 4°C in a dark glass bottle

Appendix III

PFGE Buffers And Solutions

Molecular biology grade reagents and distilled water were used wherever possible for all solutions and buffers. PFGE buffers and solutions were either autoclaved at 121°C at 120kPa or filter sterilised through a 0.22μm filter

III.i Stock Solutions

1M Tris-HCL

	Per 200ml
Tris base	24.22g

Dissolved in 150ml dH_2O with gentle heat, pH adjusted to 7.6 by addition of concentrated HCL. Made up to final volume of 200ml and stored at 4°C

0.5M EDTA

	Per litre
EDTA	186.1g
NaOH pellets	22g

Dissolved in 800ml dH_2O with gentle heat. pH adjusted to 8.0 by addition of more NaOH pellets. Made up to final volume of 1L and stored at 4°C

5M Sodium Chloride

See appendix II

20 × TE Buffer

	Per litre
Tris-HCl (200mM final conc.)	24.22g
0.5M EDTA (2mM final conc.)	4ml

Dissolved in dH_2O, pH adjusted to 8.0

10 × PFG-TBE (Tris-borate-EDTA)

	Per litre
Tris-HCL (1M final conc.)	121.1g
Boric Acid (1M final conc.)	61.83g
0.5M EDTA (2mM final conc.)	4ml

Dissolved in 800ml dH_2O with gentle heat. Made up to 1L and stored at 4°C

III.ii Specific PFGE Buffers

EC Lysis Buffer

	Per 400ml
1M Tris-HCl (6mM final conc.)	2.4ml
5M NaCl (1M final conc.)	80ml
0.5M EDTA (100mM final conc.)	80ml
0.5% Brij-58	2g
0.2% Sodium deoxycholate	0.8g
0.5% N-Lauroyl sarcosine	2g

Dissolved in 100ml dH_2O with gentle heat, Made up to 400ml and filter sterilised in 20ml aliquots. Stored at –20°C. Before use, 1mg/ml (final conc.) lysozyme and 20µg/ml (final conc.) of heat-treated RNase was added.

ESP Buffer

	Per 400ml
0.5M EDTA	400ml
1% N-Lauroyl sarcosine	4g

Sarcosine was added to sterile 0.5M EDTA solution and shaken vigorously to dissolve. 0.5mg/ml (final conc.) Proteinase K was added prior to use.

PETT IV Buffer

	Per litre
1M Tris-HCl (pH 7.6) (10mM final conc.)	10ml
5M NaCl (1M final conc.)	200ml

Made up to 1L in dH_2O and stored at 4°C

1 × TE Buffer

	Per 500ml
50 × TE buffer	25ml

Made up to 500ml in dH_2O and stored at 4°C

0.5 × PFG-TBE

	Per 2L
10 × PFG-TBE	100ml

Made up to 2L in dH_2O immediately prior to use

Appendix IV

Resistotyping Data

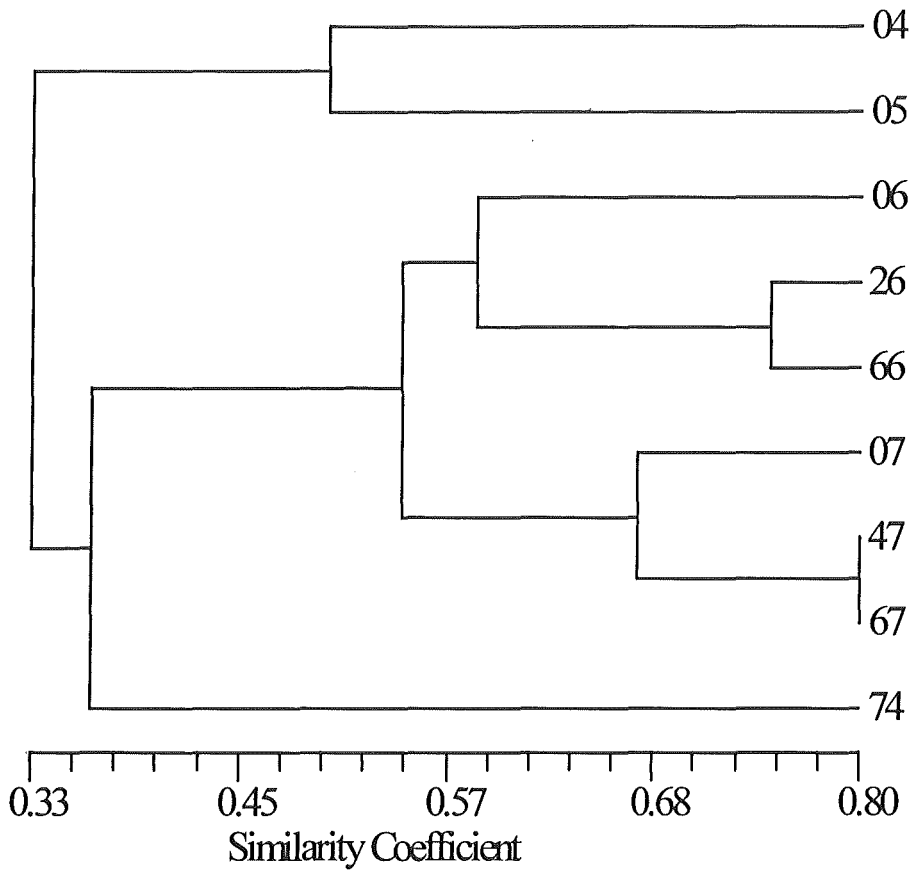

Figure A4.1: Dendrogram showing similarities between resistotype groups

Table A4.1: Size of antibiotic zones of inhibition for 22 environmental *Campylobacter* isolates

Isolate	Tetracycline hydrochloride (3 µg)		Naladixic acid (30 µg)		Metronidazole (5 µg)		2,3,5-Tetrazolium chloride (600 µg)		5-Fluorouracil (60 µg)		Sodium arsenite (20 µg)	
Replicate	1	2	1	2	1	2	1	2	1	2	1	2
AW9901	31mm	29mm	17mm	22mm	24mm	24mm	15mm	16mm	0	0	0	0
HW9902	0	0	0	0	0	0	20mm	17mm	70mm	66mm	0	0
SW9903	27mm	27mm	23mm	17mm	20mm	20mm	11mm	14mm	0	0	0	0
AW9904	24mm	26mm	17mm	21mm	18mm	16mm	0	0	50mm	46mm	0	0
AW9905	30mm	33mm	25mm	18mm	27mm	26mm	13mm	13mm	0	0	0	0
HW0006	35mm	30mm	21mm	30mm	21mm	20mm	37mm	36mm	0	0	0	0
AW0007	30mm	31mm	19mm	20mm	17mm	16mm	25mm	23mm	0	0	0	0
AW0008	25mm	22mm	0	0	15mm	13mm	22mm	21mm	0	0	0	0
EC0009	38mm	40mm	0	0	0	0	23mm	25mm	0	0	0	0
HW0010	26mm	28mm	17mm	17mm	18mm	23mm	11mm	11mm	0	0	0	0
HW0011	25mm	27mm	25mm	11mm	13mm	19mm	16mm	17mm	70mm	72mm	0	0
HW0012	14mm	13mm	0	0	0	0	0	0	0	0	0	0
GW0013	29mm	31mm	20mm	20mm	24mm	24mm	15mm	12mm	0	0	0	0
GW0014	30mm	33mm	15mm	19mm	18mm	23mm	19mm	15mm	60mm	63mm	0	0
AM0015	36mm	31mm	21mm	19mm	0	0	0	0	0	0	0	0
AM0016	31mm	33mm	30mm	30mm	20mm	23mm	17mm	18mm	44mm	46mm	0	0
AM0017	36mm	35mm	26mm	30mm	19mm	21mm	18mm	23mm	60mm	61mm	0	0
AM0018	30mm	26mm	26mm	24mm	0	0	0	0	0	0	0	0
AM0019	28mm	25mm	24mm	26mm	25mm	28mm	0	0	0	0	0	0
AM0020	25mm	28mm	30mm	25mm	19mm	20mm	0	0	0	0	0	0
AM0021	30mm	28mm	20mm	20mm	24mm	23mm	0	0	0	0	0	0
HM0022	23mm	28mm	20mm	18mm	20mm	19mm	0	0	47mm	46mm	0	0

Table A4.2: Binary data used to generate the resistotype dendrogram. A 1 indicates resistance while a 0 indicates susceptibility to the antimicrobial agent used.

Resistotype	Tet	Nal	Met	TTC	5-Fl	NaAr
04	0	0	0	0	0	1
05	0	0	0	1	0	1
06	0	0	0	0	1	1
07	0	0	0	1	1	1
26	0	1	0	0	1	1
47	0	0	1	1	1	1
66	0	1	1	0	1	1
67	0	1	1	1	1	1
74	1	1	1	0	0	1

Appendix V

FlaA RFLP Data

Table A5.1: Binary data used to generate dendrogram based on *flaA* profiles. 100-1000 = DNA fragment size in bp, 1 = Presence of a band 0 = absence of a band.

flaA type	100	125	150	175	200	225	250	275	300	325
flaA 1	1	0	1	1	0	1	0	0	1	0
flaA 2	1	0	1	0	0	1	1	0	1	1
flaA 3	0	0	1	0	0	0	0	0	0	1
flaA 4	1	0	1	0	0	1	1	0	0	0
flaA 5	1	0	1	0	0	1	0	1	0	0
flaA 6	1	0	1	0	0	0	1	1	0	0
flaA 7	0	0	0	1	0	0	1	0	0	0
flaA 8	1	0	1	0	0	1	1	0	0	0
flaA 9	1	0	1	0	0	1	0	0	0	0
flaA 10	1	1	1	0	0	1	0	0	0	0
flaA 11	1	0	1	1	0	1	0	0	0	0
flaA 12	1	0	1	1	0	1	1	0	0	0
flaA 13	1	0	1	1	0	1	1	0	0	0
flaA 14	1	0	1	0	0	0	1	0	1	0
flaA 15	1	0	1	0	1	0	0	1	0	0
flaA 16	1	0	1	0	0	1	1	0	0	0
flaA 17	0	1	0	1	0	1	0	0	0	1

Table A5.1: Continued....

flaA type	350	375	400	425	450	475	500	525	550	575	600	625	650
flaA 1	0	0	0	0	0	0	0	0	0	0	0	0	0
flaA 2	0	0	0	0	0	0	0	0	0	0	0	0	0
flaA 3	0	0	0	0	1	0	0	0	0	0	0	0	0
flaA 4	0	0	0	0	0	0	0	0	0	0	0	0	0
flaA 5	1	0	0	1	0	0	1	0	0	0	0	0	0
flaA 6	0	0	0	0	0	0	0	0	0	0	0	0	0
flaA 7	0	0	0	0	1	0	0	0	0	0	0	0	1
flaA 8	0	0	1	0	0	1	0	0	0	0	0	0	0
flaA 9	1	0	0	0	0	0	0	0	1	0	0	0	0
flaA 10	0	0	0	0	0	0	1	0	0	0	0	0	0
flaA 11	0	0	0	0	0	0	0	0	0	0	0	0	0
flaA 12	0	1	0	0	0	0	0	0	0	0	0	0	0
flaA 13	0	0	0	0	0	0	0	0	0	1	0	0	0
flaA 14	1	0	0	0	0	0	1	0	0	0	0	0	0
flaA 15	0	0	0	1	0	0	0	0	0	0	0	0	0
flaA 16	0	0	0	0	0	0	0	0	0	0	0	0	0
flaA 17	0	1	0	0	0	0	0	0	0	0	0	0	0

Table A5.1: Continued....

flaA type	675	700	725	775	800	825	850	875	900	925	950	975	1000
flaA 1	0	0	0	0	0	0	0	0	0	0	0	0	0
flaA 2	0	0	0	0	0	0	0	0	0	0	0	0	0
flaA 3	0	0	0	0	0	0	0	0	0	0	0	0	0
flaA 4	0	0	0	0	0	0	1	0	0	0	0	0	0
flaA 5	0	0	0	0	0	0	0	0	0	0	0	0	0
flaA 6	0	0	0	0	0	0	0	0	0	0	0	0	1
flaA 7	0	0	0	0	0	0	0	0	0	0	0	0	0
flaA 8	0	0	0	0	0	0	0	0	0	0	0	0	0
flaA 9	0	0	0	0	0	0	0	0	0	0	0	0	0
flaA 10	0	0	0	0	0	0	0	0	0	0	0	0	0
flaA 11	0	0	0	0	0	0	0	0	0	1	0	0	0
flaA 12	0	0	0	0	0	0	0	0	0	0	0	0	0
flaA 13	0	0	0	0	0	0	0	0	0	0	0	0	0
flaA 14	0	0	0	0	0	0	0	0	0	0	0	0	0
flaA 15	0	0	0	0	0	0	0	0	0	0	0	0	0
flaA 16	0	0	0	0	0	0	0	0	0	0	0	0	0
flaA 17	0	0	0	0	0	0	0	0	0	0	0	0	0

Appendix VI

PFGE Data

Table A6.1: Binary data used to generate dendrogram based on PFGE profiles. 48.5-436.5 = DNA fragment size in kb, 1 = Presence of a band 0 = Absence of a band

PFGE type	48.5	58.2	67.9	77.6	87.3	97	109.1	121.3	133.4	145.5	157.6
PFGE-1	0	0	0	1	0	0	0	1	1	1	1
PFGE-2	0	1	0	1	0	0	0	1	1	1	1
PFGE-3	0	0	0	0	0	0	0	1	1	0	0
PFGE-4	1	0	0	0	0	0	1	1	0	0	1
PFGE-5	0	0	0	0	1	0	0	1	1	1	1
PFGE-6	0	0	0	0	0	0	1	0	1	0	0
PFGE-7	0	0	1	0	0	0	0	0	1	1	1
PFGE-8	1	0	0	1	1	0	0	0	0	0	0
PFGE-9	0	0	0	1	0	0	0	1	1	1	1
PFGE-10	0	0	0	1	0	0	1	0	1	1	1
PFGE-11	0	0	0	1	0	0	0	0	1	1	1
PFGE-12	0	0	0	0	0	1	1	1	1	0	1
PFGE-13	0	0	0	0	1	0	0	1	0	1	1
PFGE-14	0	0	0	0	0	0	0	1	1	0	0
PFGE-15	0	0	0	1	0	0	1	1	1	1	1

Table A6.1: Continued...

PFGE type	169.8	181.9	194	210.2	226.3	242.5	258.7	274.8	291	315.3	339.5
PFGE-1	0	0	0	0	0	0	0	0	0	0	1
PFGE-2	0	0	0	0	0	0	0	0	0	0	1
PFGE-3	0	0	0	0	0	0	0	0	0	1	0
PFGE-4	0	0	0	1	0	0	0	0	0	1	0
PFGE-5	0	0	0	0	0	0	0	0	0	0	1
PFGE-6	0	0	0	0	0	1	0	0	0	0	0
PFGE-7	0	1	0	0	0	0	0	0	1	1	0
PFGE-8	1	0	0	0	0	0	0	1	0	0	0
PFGE-9	0	0	0	0	0	0	0	0	1	0	0
PFGE-10	0	0	0	0	0	0	0	1	0	0	0
PFGE-11	0	0	0	0	0	0	0	0	1	0	0
PFGE-12	0	0	0	0	0	0	0	1	0	1	0
PFGE-13	0	0	0	0	0	0	0	0	1	0	0
PFGE-14	0	0	0	0	0	0	0	0	1	0	0
PFGE-15	0	0	0	0	0	0	0	0	0	0	1

Table A6.1: Continued...

PFGE type	363.8	388	412.3	436.5
PFGE-1	0	0	0	0
PFGE-2	0	1	0	0
PFGE-3	1	0	0	0
PFGE-4	0	0	0	0
PFGE-5	0	0	0	0
PFGE-6	1	0	0	0
PFGE-7	0	0	1	0
PFGE-8	0	0	0	1
PFGE-9	0	1	0	0
PFGE-10	1	0	0	0
PFGE-11	1	0	0	0
PFGE-12	0	0	0	0
PFGE-13	0	0	0	0
PFGE-14	1	1	0	0
PFGE-15	0	0	0	0